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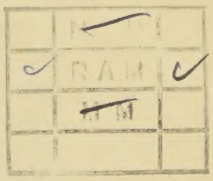

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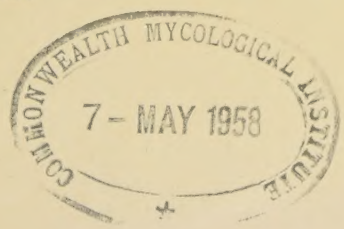

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ACCUMULATION OF ALLO-ISOCITRIC ACID BY A PENICILLIUM STRAIN

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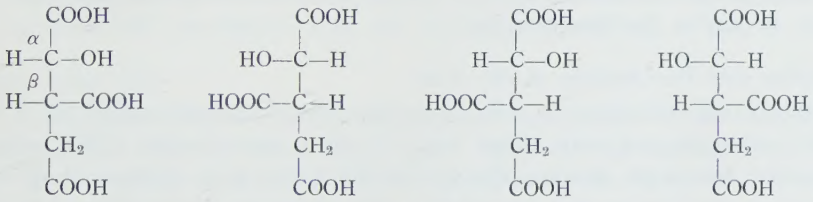
The natural occurrence of optically active isocitric acid was first demonstrated by Nelson[1] and Bruce[2] in blackberry fruits, and a somewhat large accumulation of the acid was found by Pucher et al.[3] in Bryophyllum leaf tissue. This acid has also been recognized as a member of the tri-carboxylic acid cycle in many animals, plants and microorganisms. All isocitric acid, found before in nature, was the same stereoisomer, the free acid of which was dextrotatory in aqueous solution and its lactone having strong levorotatory activity. Therefore this has been given the prefix of *d*-.

Since isocitric acid contains two asymmetric centers, its diastereoisomer must exist, i.e., allo-isocitric acid, and then, the occurrence of four stereoisomers, i.e., *d*-, *l*-isocitric acid and *d*-, *l*-allo-isocitric acid, would be expected. The presence of *dl*-allo-isocitric acid in the synthetic acid was first demonstrated by Pucher et al.[4], but they could not obtain it in a pure state.

Recently, the stereochemistry of the α-carbon of isocitric acid was elucidated by Greenstein et al.[5],[6]. All four stereoisomers were obtained in a pure state and configuration of the α-carbon of these isomers were decided. *d*-Isocitric acid was proved to belong to the L$_s$-series.



Though participation of isocitric acid in the tri-carboxylic acid cycle in microorganisms is apparent, its accumulation by microorganisms has yet never been reported. The authors have found that large amounts of isocitric acid is accumulated in a glucose medium by a fungus, *Penicillium purpurogenum* var. and further studies indicated that the accumulated acid is *d*-allo-isocitric acid. Referring to the stereochemical studies by Greenstein et al.[5],[6], the isolated acid was ascertained to be L$_s$-allo-isocitric acid.

* Kyōwa Fermentation Ind. Co. Ltd.

The presence of allo-isocitric acid in nature has heretofore never been reported.

EXPERIMENTAL

Fermentation Experiments

The strain *Penicillium purpurogenum* Stoll var. *rubri-sclerotium* Thom (non-sclerotigenic type) No. 1148[7] was used. This organism was grown in a medium with the following composition; 3 g peptone, 0.015 g KH_2PO_4 and K_2HPO_4, 0.01 g $MgSO_4 \cdot 7H_2O$ and $CaCl_2$, trace of $FeSO_4$ and NaCl, made up to 100 ml with distilled water, and 1% dry sterilized $CaCO_3$ was added aseptically to the autoclaved medium.

Cultivation was performed aerobically by shaking culture at 30°C, for 4–7 days.

The accumulated acids were extracted by continuous ether extraction and chromatographed on Toyô No. 50 filter paper using a mixture of *n*-butanol 4: formic acid 1.5: water 1 (v/v) as the ascending solvent. Two large acidic spots, *Rf* 0.55 and 0.40, were observed, but after heating with excess alkali, the aqueous solution of the extracts gave only one spot, *Rf* 0.40. This indicated that the acid of *Rf* 0.55 might be the lactone of the above acid of *Rf* 0.40, and this was ascertained by further studies.

The following facts were observed. Ether-insoluble acids were not detected by paper chromatography. Citrate was not proved by the pentabromoacetone test. No volatile acid was detected. Thus the acid of *Rf* 0.40 was supposed to be only one acidic product of this fungus at the given condition.

Addition of $CaCO_3$ is essential for large accumulation of the acid, and in the absence of $CaCO_3$, the amount of the accumulated acid is less than one-tenth of that of the former case.

Isolation and Purification of the Acid

After the filtration, a culture medium was acidified below pH 1 with H_2SO_4 and extracted with ether for 3–7 days continuously. The extracts contained two acids, that is, the acid of *Rf* 0.40 and its lactone of *Rf* 0.55. Therefore purification was undertaken after complete dehydration of the acid to its lactone.

The ether extract was treated with activated charcoal and evaporated to a sirup which was then heated at 100° in vacuo so as to dehydrate the acid. The sirup solidified slowly, and then the resulting cake was crushed and heated at 100° till it became entirely dry. Paper chromatograph of this crude powder gave only one spot, *Rf* 0.55. It was washed twice with a small amount of ether and then dissolved to possibly small amount of hot ethyl acetate. Chloroform was added dropwise to this solution till cloudiness appeared, and the solution was chilled slowly. The resulting crude crystal

of the lactone could be purified by repeated crystallization from ethylacetate-chloroform.

Analysis and Properties of the Isolated Lactone (I)

The pure lactone sinters at 137° and melts at 141–142° to yellow oil.

Anal. Calcd. for $C_6H_6O_6$: C, 41.33; H, 3.47. Found: C, 41.24; H, 3.54
$[\alpha]_D^{19°}$, +42.3° (c=4.83, H_2O).

The lactone was titrated potentiometrically as a dibasic acid with pK_{a1} 2.4 and pK_{a2} 3.7 in cold water, and a neutralization equivalent of 88.2 was observed: Calcd. for isocitric lactone; 87.03. But after treatment with excess alkali at 100° for 10 minutes so as to open the lactone ring, one more acidic group appeared by back-titration. An equivalent weight observed was 177.5, calcd.; 174.1.

The infrared spectrum of the lactone in Nujol mull showed a strong broad absorption at 5.75 μ and sharp one at 5.88 μ, which probably referred to the C-O- stretching vibration of γ-lactone ring and C=O stretching of carboxyl group, respectively.

These results agreed with isocitric γ-lactone.

Methyl ester of the Lactone (II)

An ethereal solution of the lactone was treated with diazomethane, and resulting methylester was recrystallized from absolute ethanol, m.p. 49–50°. It was supposed to be the dimethylester of the isolated lactone.

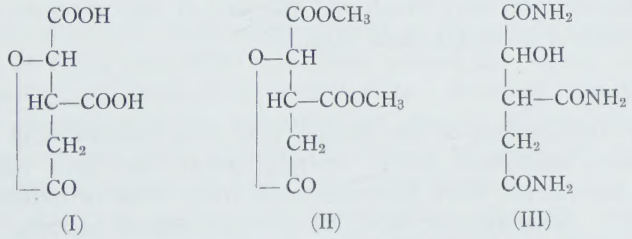
Anal. Calcd. for $C_8H_{10}O_6$: C, 47.53; H, 4.99. Found: C, 47.80; H, 5.34
$[\alpha]_D^{22°}$, +56.2° (c=0.85, methanol)

Molecular weight (determined by Rast's method): Calcd. for $C_8H_{10}O_6$; 202. Measured; 199

The infrared spectrum of the methylester in chloroform solution indicated the presence of γ-lactone (5.60 μ) and methyl ester (5.79 μ). (Fig. 4)

Acid amide (III)

A solution of 1 g of the dimethylester of the lactone in 5 ml of methanol was saturated with dry ammonium gas. Heat was spontaneously evolved and upon standing over night, a white precipitate was deposited. The sub-



(I) (II) (III)

Fig. 1. Derivatives of isocitric acid.

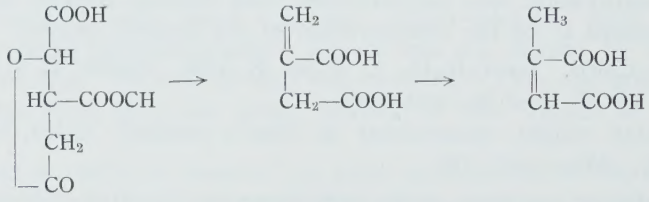
stance was dissolved slowly in water at 45° and precipitated on cooling to 0°. It melted at 189° with decomposition. It was supposed to be a tri-acidamide of the acid.

Anal. Calcd. for $C_6H_{11}O_4N_3$: C, 38.09; H, 5.88; N, 22.20. Found: C, 38.27; H, 5.58; N, 21.70.

Degradation Experiments

Heat degradation products of the isolated lactone also agreed with isocitric lactone. By dry distillation of the lactone, colourless oily products, probably acid anhydrides, were obtained. This substance gave positive tests for olefinic unsaturation, and after treating with hot water, almost only citraconic (V) (*Rf* 0.80) and itaconic acids (IV) (*Rf* 0.89) were detected by paperchromatography using the same solvent mentioned above.

Furthermore, itaconic acid was isolated from this mixture by silica gel chromatography. Columns of 33 mm in diameter were packed with 30 g of Mallinckrodt's chromatographic silicic acid retaining 22 ml of 0.5 N-H_2SO_4 as the stationary phase. As the developing solvent, 3% *n*-butanol (v/v) in benzol was used. The peak eluentes were collected and the solvent was evaporated in vacuo. By this method, itaconic acid was obtained in crystalline form (m.p. 158–160°). Admixture with an authentic itaconic acid, m.p. 160°, showed no melting point depression (m.p. 158–160°).



(IV) (V)

Fig. 2. Heat-degradation of isocitric lactone.

Infrared spectrum of the Tri-sodium salt

Identity with isocitric acid was proved by the infrared spectrum. The spectrum of the tri-sodium salt of the isolated acid in Nujol mull was almost completely identical with that of an authentic specimen (obtained from Nutritional Biochemical Co.) which was the salt of the racemic acid corresponding to natural *d*-isocitric acid (Fig. 3).

Stereoisomerism

Although the identity of the isolated acid with isocitric acid was proved from the results mentioned above, melting points and $[\alpha]_D$ values of the acid and its derivatives were quite different from those of natural *d*-isocitric acid (Table 1). Since isocitric acid has two asymmetric centers, the isolated acid is supposed to be its diastereoiomer, i.e., allo-isocitric acid. This fact

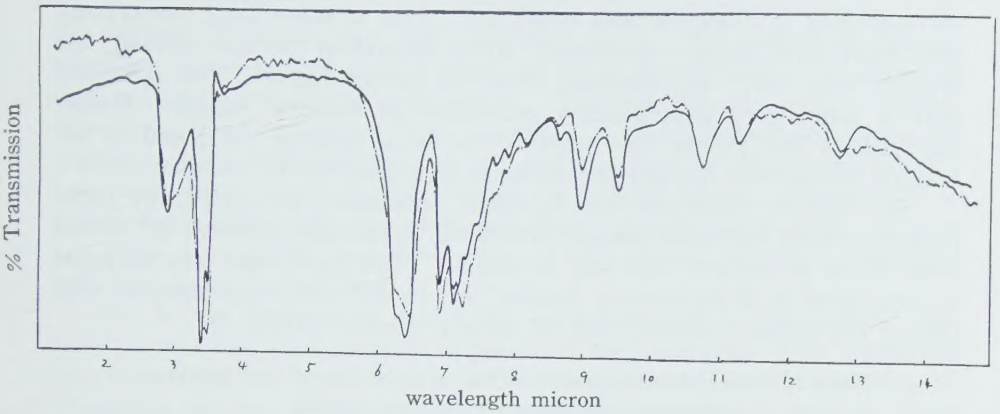


Fig. 3. Infrared spectrum of the tri-sodium salt of the sample.
solid line: trisodium salt of the sample.
broken line: 〃 of *dl*-isocitric acid.

was identified with the synthetic *dl*-allo-isocitric acid by both the paper chromatograph and the infrared spectrum.

Table 1. Melting points and $[\alpha]_D$ values of derivatives of the isolated acid and *d*-isocilated acid.

	The isolated acid		*d*-isocitric acid	
	m.p.	$[\alpha]_D$	m.p.	$[\alpha]_D$
Free acid		+36.8°a)		−17.7°a)
γ-lactone	141–2°	+42.3°	156–7°	−62.0°
〃 -dimethyl-ester	49–50°	+56.2°b)	106°	−63–5°b)
Tri-acid amide	189°c)		204°c)	− 5.02°

a) $[\alpha]_D$ values of the free acid measured after acidification of aqueous solution of the tri-sodium salt with HCl.
b) $[\alpha]_D$ in methanol.
c) m.p. with decomposition.

The occurrence of *dl*-alloisocitric acid in the substance synthesized by the method of Fittig and Miller has been proved by Pucher and Vickery, but they could not obtain it in a pure state. Recently, Senear[9] obtained the pure *dl*-allo-acid from the epimerization product of isocitric acid. The authors obtained the pure allo-isocitric lactone by silica gel chromatograph from the synthetic product of the Fittig-Miller method.

Isocitric acid was synthesized from sodium succinate and anhydrous chloral by the method originally described by Fittig and Miller and further modified by Pucher et al. The racemic isocitric lactone, m.p. 160–161°,

corresponding to *d*-isocitric acid which was found in plant tissue was crystallized from ethyl acetate-chloroform. After separation of this lactone, the crystal, m.p. 138°, was obtained from the mother liquor. This substance gave a satisfactory neutralization equivalent as isocitric lactone. Pucher and Vickery had proved this material to be a mixture composed of *dl*-isocitric lactone and considerable amounts of *dl*-allo-isocitric lactone.

The authors examined this synthetic substance, m.p. 138°, by paper chromatography and found the spot which had apparently different *Rf* values from these of isocitric acid and its lactone. The above spot was supposed to correspond to *dl*-allo-isocitric lactone, and its *Rf* value was identical with that of the lactone isolated from the culture medium (Table 2).

Table 2. Paper chromatography of the isolated lactone and synthetic substance containing allo-isocitric lactone.

Solv. *n*-BuOH:Formic acid:Water=4:1.5:1 (v/v)	
Rf value of the isolated lactone	0.69
〃 of the synthetic substance containing allo-lactone (three spots)	0.60 0.69 0.91*
〃 of *dl*-isocitric lactone	0.60

* A very slight amount, probably due to polymerization.

Final identification with this synthetic allo-lactone was made after the esterification by the infrared spectrum of the lactone-dimethylester in a chloroform solution.

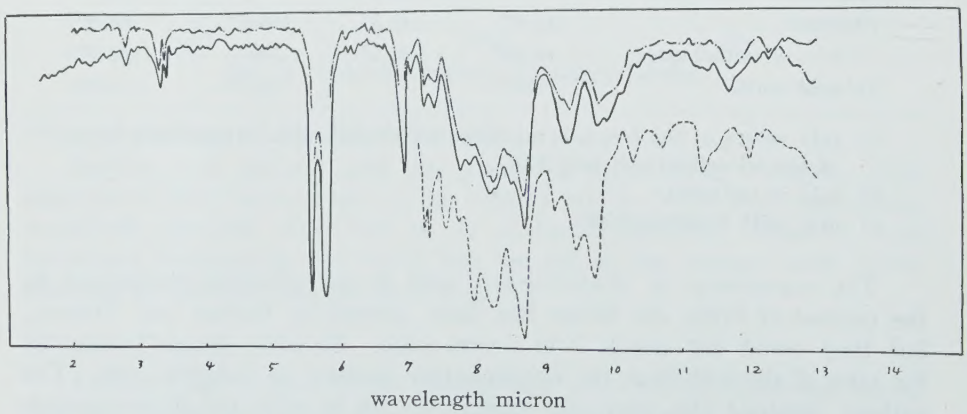


Fig. 4. Infrared spectruum of the lactone-dimethyl ester in chloroform solution.

solid line: Lactone-dimethylester of the sample
broken line: 〃 of the synthetic *dl*-allo-isocitric acid
dotted line: 〃 of *dl*-isocitric acid

dl-Allo-isocitric lactone was isolated by silica gel chromatography from the synthetic substance, m.p. 138°, described above. The method of chromatography was the same as that described above except using 10% *n*-butanol (v/v) in benzol as the developing solvent. The resulting allo-isocitric lactone, free from isocitric lactone, was converted to the lactone-dimethylester by treatment with diazomethane. Its infrared spectrum in chloroform solution was completely identical with that of the isolated lactone, whereas that of the synthetic *dl*-isocitric lactone dimethylester differed considerably. (Fig. 4)

From the results described above, it was proved that the accumulated acid was *d*-allo-isocitric acid.

As it has been noted above the tri-sodium salt of the isolated allo-isocitric acid had shown almost identical infrared spectrum with tri-sodium isocitrate which had been the salt of the racemate of *d*-isocitric acid (Fig. 3). Therefore the ring formation of the laotone probably caused increase of the difference in spectrum between these two diastereoisomers.

Table 3. Melting points and $[\alpha]_D$ values of the four stereoisomers of isocitric lactone and *d*-allo-isocitric lactone accumulated by the fungus.

		m.p.	$[\alpha]_D$	Reported by,
Allo-isocitric lactone	DL	153°		Greenstein et al.
	D	143°	−40.5°	
	L	143°	+41.0°	
Isocitric lactone	DL	161°		
	D	154°	+60.9°	
	L	154°	−61.1°	
d-isocitric lactone		153–4°	−62.0°	Bruce
Allo-isocitric lactone accumulated by the Penicillium.		141–2°	+42.3°	

Recent stereochemical studies by Greenstein et al., decided the configuration of α-carbon of isocitric acid and showed m.p. and $[\alpha]_D$ values of all the four stereoisomers of this acid. These values of the isolated lactone agreed well with L$_s$-allo-isocitric lactone (Table 3). Admixture with the latter lactone which had been kindly presented by Dr. J. P. Greenstein showed no melting point depression (m.p. 141–142°).

Because "natural" *d*-isocitric acid has been proved to equally belong to the L-series, it is apparent that allo-isocitric acid accumulated by this fungus has the same configuration of the α-carbon with the former.

Yield of the acid

Allo-isocitric acid was the only one detectable acid accumulated by the fungus in the given condition throughout the cultivation period. Therefore

the amount of the acid mentioned was calculated from its total acidity. The yield of the acid (by weight) exceeded 70% of the consumed glucose at 6 day's shaking cultivation.

SUMMARY

The occurrence of L$_s$-allo-isocitric acid in nature, as a new fugal product, was first found in the culture medium of *Pen. purpurogenum* Stoll var. *rubri-sclerotium* Thom. No. 1148. The acid was obtained as its γ-lactone in a crystalline form.

Some chemical and physical characters were described.

The acid was the only one detectable acid accumulated by this fungus in the given condition, and the yield of the acid by weight exceeded about 70% of the consumed glucose.

ACKNOWLECGEMENTS

The authors are deeply indebted to Dr. J. P. Greenstein of the National Cancer Institute, National Institute of Health, U.S.A., for supplying with synthetic L$_s$-allo-isocitric lactone. Their thanks are also due to Dr. K. Tsuda of the Institute of Applied Microbiology, University of Tokyo, and Dr. M. Matsui of Department of Agricultural Chemistry, University of Tokyo, for kind advice and suggestions.

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STUDIES ON THE METABOLISM OF D-AMINO ACID IN MICROORGANISMS

PART 4. ON THE NATURE OF THE PURIFIED D-GLUTAMIC-ASPARTIC OXIDASE OF *ASPERGILLUS USTUS*

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It has been shown that the enzyme perparation responcible for the oxidation of D-glutamic and D-aspartic acids can be obtained from *Aspergillus ustus strain f* isolated from the soil (1). With the use of a partially purified enzyme preparation, it has also been shown that D-glutamic and D-aspartic acids were oxidized by a single enzyme, and this enzyme has been called D-monoaminodicarboxylic acid oxidase (2). The purification of the enzyme has been continued and a highly purified enzyme preparation (approximately 90% pure) has been obtained. This preparation can oxidize both D-glutamic and D-aspartic acids at the same rate, but cannot oxidize other D-monoaminodicarboxylic acids such as α-aminoadipic acid and α-aminomalonic acid. Therefore, the name D-glutamic-aspartic oxidase seems to be more appropriate for this enzyme than its previous name, i.e., D-monoaminodicarboxylic acid oxidase. Hereafter, the name D-glutamic-aspartic oxidase will be employed for the enzyme instead of its previous name.

The present paper is concerned with further purification procedures of the enzyme and the nature of the prosthetic group.

MATERIALS AND METHODS

Chemicals. The followings are the reagents used and their source: Flavin adenine dinucleotide (FAD) (85% pure) prepared from *Eremothecium ashibyii* (3) and authentic flavin mononucleotide (FMN) (95% pure). Both of these samples were kindly provided by Dr. Y. Matsuoka. D-Glutamic acid was a sample kindly supplied from Ajinomoto Co. Ltd. DL-α-Aminoadipic acid and α-aminomalonic acid were kindly furnished by the Laboratory of Biochemistry, Faculty of Agriculture, University of Tôhoku.

Borate phosphate buffer (pH 8.0) was prepared by mixing 10.20 gr of $Na_2B_4O_7$ and 6.33 gr of KH_2PO_4 in 1 litre of distilled water.

Calcium phosphate gel was prepared by the following method: To 1 litre of 0.44 M Na_2HPO_4 was added 1 litre of 0.66 M $CaCl_2$ with vigorous stirring in the cold. After adjusting the pH of the mixture solution to 8.5 with

a concentrated ammonium solution, the gel was recovered by low-centrifugation and washed with distilled water until the supernatant fluid showed no trace of the Nessler reaction.

Estimation of active flavin content. The active flavin content of the enzyme was determined by the ΔE_{450} on reduction with D-glutamate.

Estimation of metals in the enzyme. The amount of metals was semi-quantitatively analyzed using an Adam Hilger E-2 type Medium Quartz Spectrograph.

Enzymatic assay and definition of Specific activity. Oxidase activity was estimated manometrically in a Warburg vessel at 30°C. Specific activity was defined as oxidase activity (Qo_2) per mg protein-N. Protein-N was determined by the micro-kyeldahl method after dialyzing a sample against running water for 48 hours.

Medium. Aspergillus ustus strain f was grown in a medium containing crude glucose (70% pure) 2%, crude D-glutamic acid (90% pure) 0.5%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1% and a trace of $FeSO_4 \cdot 7H_2O$ in a one to one mixture of distilled water and tap water.

Cultivation. The organisms were grown in a fermenter containing 200 litres of the medium and incubated at 30°C with aeration (200 litres per minute). The organisms were harvested at the latter period of the logarithmic growth phase.

PREPARATION OF ENZYME

Step 1. Extraction of enzyme. Three kilogrames of the wet mycelium of *Aspergillus ustus strain f* was obtained from the four days' culture, aerobically grown in 100 litres of the medium. The mycelium was ground in a mortar with aluminum oxide and was eluted twice with borate phosphate buffer through a cotton cloth. To the extracts was added 70 gr of activated carbon. This was allowed to stand for 30 minutes with occasional stirring and centrifuged to remove activated carbon. By these procedures 8 litres of the colorless crude extracts was obtained.

Step 2. Absorption and elution of enzyme from calcium phosphate gel. To the crude extracts was added wet calcium phosphate gel (70 gr dry-weight), and the pH of the mixture solution was adjusted to 6.0. After standing for 30 minutes, the clear supernatant solution was removed by decantation, and from the residual gel suspensin the gel precipitates were collected by low-centrifugation followed by washing with distilled water. Then the precipitates were mixed with 400 ml of borate phosphate buffer and the pH of the mixture was adjusted to 7.5. After standing for 15 minutes the eluate was recovered by low centrifugation. The same eluting procedures with a portion of 200 ml of the buffer were repeated twice; thus, an amount of 800 ml of the eluate was obtained.

Step 3. First fractionation with ammonium sulfate. To the enzyme

solution solid ammonium sulfate was added to 43% saturation at 2°C. The precipitate was removed by centrifugation and with addition of ammonium sulfate, the supernatant solution was adjusted to 53% saturation. The resultant precipitates were recoved by centrifugation, dissolved in 50 ml of borate phosphate buffer and dialyzed against the same buffer.

Step 4. Second fractionation with ammonium sulfate. Fractionation procedures similar to step 3 were carried out. The recovered precipitates were dissolved in 2 ml of M/12-sodium succinate buffer (pH 5.35) and dialyzed against the same buffer.

Step 5. Column chromatographic purification on ion exchange resin. The dialyzed preparation was applied to a column of XE-64 (0.9 cm×7.0 cm) which had been equilibrated with M/12-sodium succinate buffer at pH 5.35. After rinsing the sample with the buffer, 30 ml of the buffer was passed through the column at a rate of 10 ml per hour. Then a buffer of pH 5.60 was added until the yellowish band was run off the column. The elution curve is shown in Fig. 1. The eluates in the tubes No. 15 to 17 were again salted out with

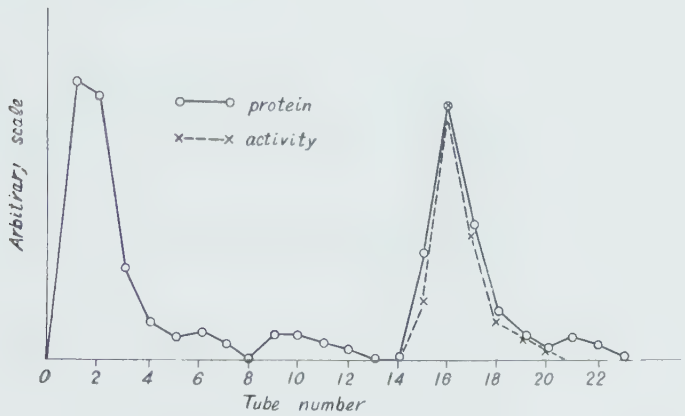

Fig. 1. Chromatography of the enzyme on a column of XE-64. Amount of protein was estimated by Folin's method. Enzyme activity towards D-glutamate and D-aspartate was estimated by oxygen consumption.

ammonium sulfate and the precipitates were dissolved in 1 ml of M/5-Tris buffer (pH 8.0) and dialyzed against the same buffer.

Throughout these procedures approximately 10% of the enzyme was recovered in the final purified preparation.

Purity of the enzyme preparation. The over-all purification was 500-fold compared with the crude extracts and the Q_{O_2} of the final preparation was 150,000 for both D-glutamic and D-aspartic acids at 30°C. Only a trace of catalase activity was detected in the final enzyme preparation. Examination on an analytical ultracentrifuge (Fig. 2) revealed that the enzyme preparation had a high degree of homogeneity (approximately 90% pure).

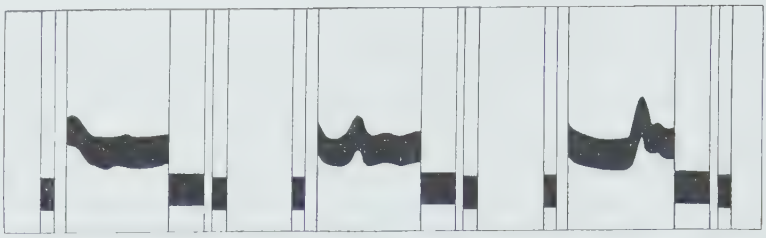

Fig. 2. Sedimentation pattern of purified enzyme in an ultracentrifuge (Spinco Model-E)
Buffer, 0.2 M tris buffer pH 8.0, temperature 4.4°, speed 59800 r.p.m., bar angle 55°, Each of the three exposure was spaced 32 minutes apart. The yellowish portion was always kept at the left of the main boundary.

RESULTS AND DISCUSSION

Substrate specificity. This enzyme showed a high specificity towards D-glutamic and D-aspartic acids and could oxidize both D-amino acids at the same rate at the substrate concentration of 2×10^{-2} M, where these D-amino acids were saturated for the enzyme. The enzyme showed no activity towards DL-α-aminoadipic acid, α-aminomalonic acid and all of the other DL-amino acids tested. In addition, DL-α-aminoadipic acid and α-aminomalonic acid did not compete with D-glutamic acid and D-aspartic acid for the active site on the enzyme.

Prosthetic group. The ultraviolet absorption spectrum of the enzyme is shown in Fig. 3A. The appearance was that of a typical flavoprotein with

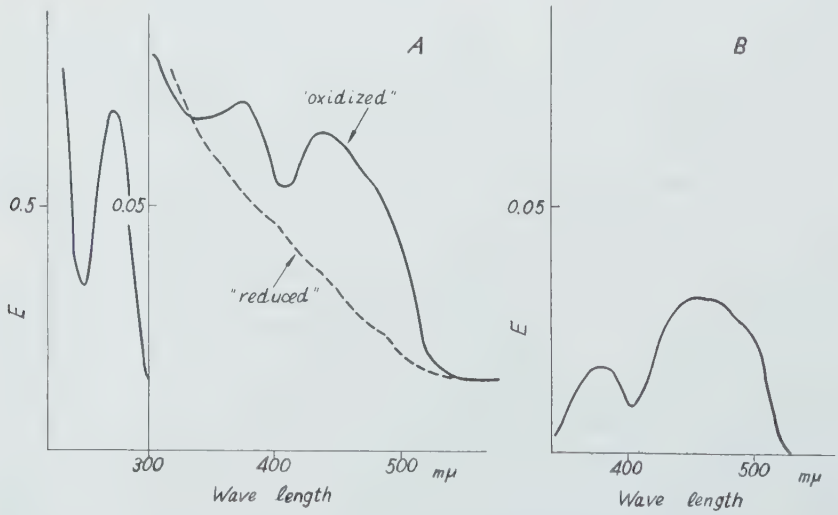

Fig. 3A. Absorption spectrum of D-glutamic-aspartic oxidase. The tracing marked "reduced" was obtained after the addition of 40 μM of D-glutamate.
B. Difference of spectrum (oxidized minus reduced) of the enzyme.

maxima at 278, 370 and 440 mμ. The enzyme was rapidly reduced by D-glutamate and the spectrum of the enzyme treated in such a manner showed no peak at 440 and 370 mμ. The differences of the spectra are presented in Fig. 3B. This shows a typical absorption spectrum of flavin compound suggesting that the prosthetic group of the enzyme is a flavin nucleotide. When the enzyme was salted out with ammonium sulfate and the pH of the mixed solution was adjusted to 1.6, the flavin portion of the enzymd was reversibly released without denaturation of the protein portion. The activity of the enzyme thus obtained, was greatly reduced but could be restored by the addition of FAD as shown in Table 1. The addition of FMN was rather

Table 1. Effect of Flavin Compounds on the Acid-Treated Enzyme

Additions	O_2 uptake in 20 min.
Enzyme	0 μl
Enzyme+D-glutamate	28
Enzyme+D-glutamate+FAD (20 γ)	188
Enzyme+D-glutamate+FMN (20 γ)	10

detrimental. This inhibitory action of FMN would probably be caused by its competitive action with FAD for the active site on the enzyme. The amount of active flavin in the enzyme was calculated from the difference of its optical density at 450 mμ. The turnover number of the enzyme could, therefore, be estimated to be about 5000 per minute per mole of flavin. Though the FAD used in this experiment contained some impurities, no significant amounts of other flavin compounds were detected in it. Therefore, there is no possibility that some impurities found in the FAD preparation would be a true prosthetic group. As described in the previous report (1) the enzyme was so stable that storage at −20°C for a few months was possible. However, when the flavin portion (FAD) was removed from the enzyme, the enzyme became very labile as demonstrated in Table 2. This

Table 2. Stability of Apo- and Holo-Enzymes

	Addition	Immediately after acid-treatment	Storage at 0°, 4 hr. after acid-treatment
(1)	Enzyme+D-glutamate	72 μl	70 μl
(2)	Enzyme+D-glutamete+FAD	420	260
(3)	Effect of FAD (2)−(1)	348	190

(1) shows activity of flavin conjugated enzyme in the acid-treated enzyme preparation.
(3) corresponds to the amount of active apo-enzyme.

fact suggests that FAD is indispensable not only as a co-enzyme but also as a stabilizing material for the enzyme.

The existence of some metals in the enzyme was presumed because of the remarkable inhibitory effect of cyanide on enzyme action. However, as shown in Table 3, significant amounts of any metals are not to be detected

Table 3. Relative Amount of Flavin and Metals Contained in the Enzyme

Component	Weight-ratio	Molecular-ratio
FAD	1	1
Mo	<0.0004	<0.003
Fe	<0.004	<0.06
Cu	<0.01	<0.12

in the enzyme by the spectroscopic method.

Specificity of electron acceptor. In the presence of the purified enzyme, D-glutamic acid could be oxidized by molecular oxygen, 2,6-dichlorophenol-indophenol, methylene blue or ferricyanide, but could not be oxidized by cytochrome c or triphenyltetrazolium chloride even in the presence of metals such as Mo, Cu and Fe. The relative rate of oxidation by these electron acceptors are shown in the order as follows:

$$\text{oxygen} > \text{ferricyanide} > \text{indophenol} > \text{methylene blue}$$

Mechanisms of KCN inhibition. As previously described, the enzyme action was remarkably inhibited in the presence of KCN at the concentration of 10^{-3} M. The following phenomena concerning the mechanisms of KCN inhibition on the enzyme action were observed.

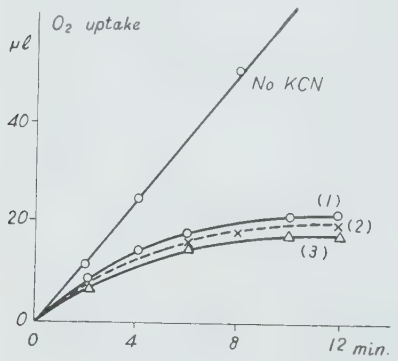

Fig. 4. Effect of KCN on the oxidation of D-glutamate. Concentration of KCN was 10^{-2} M in (1), (2) and (3). Preincubation time: (1) 5 min., (2) 10 min., (3) 30 min.

1. The effect of KCN gradually appeared after the enzymation was started, regardless of the time of preincubation with the enzyme (Fig. 4).

2. The addition of α-ketoglutarate and hydrogen peroxide to the reaction mixture did not have effect on the pattern of KCN inhibition. This shows that inhibition was not caused by the presece of the reaction product, α-ketoglutarate nor hydrogen peroxide.

3. When 2,6-dichlorophenol-indophenol was used as a electron acceptor, only a very slight inhibitory effect was observed with KCN. However, when the enzyme had been pretreated with KCN and D-glutamate at 30°C for several minutes in aerobic condition, a remarkable inhibition was observed even when indophenol was used as an electron acceptor.

From these results it was concluded that oxygen and the substrate (D-glutamate or D-aspartate) would be indispensable for the inhibitory action of KCN on the enzymatic reaction. Studies on the roles of these substances in this inhibitory action are now in progress.

As a result of these studies, it appeared that the enzyme is a conjugated flavoprotein which has a FAD as the sole prosthetic group. The facts that significant amounts of the metallic component are not detectable in the enzyme nor is it capable of interaction with cytochrome c even in the presence of metallic carriers such as Fe, Cu or Mo, indicate the complete difference of this enzyme from metalloflavoprotein. The specificity of the enzyme is strictly confined to D-glutamic and D-aspartic acids. Therefore, this enzyme should be called D-glutamic-aspartic oxidase instead of D-monoaminodicarboxylic acid oxidase.

SUMMARY

1. Further purification of the enzyme responsible for the oxidation of D-glutamic and D-aspartic acids was carried out and an enzyme of 90% purity was obtained.

2. This enzyme could oxidize D-glutamic and D-aspartic acids but was incapable of oxidizing DL-α-aminoadipic and α-aminomalonic acids. The name D-glutamic-aspartic oxidase was designated for this enzyme instead of its previous name, D-monoaminodicarboxylic acid oxidase.

3. The prosthetic group of the enzyme was proved to be FAD.

4. No metallic component was detectable in the enzyme.

5. Some studies concerning the mechanisms of the inhibitory action of KCN are also presented here.

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AN IMPROVED METHOD FOR ISOLATING BIOCHEMICAL MUTANTS OF *STREPTOMYCES GRISEOFLAVUS*

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Studies concerning the genetics of *Streptomyces* have been greatly developed in the past two years, by the discovery of genetic recombination in *Str. coelicolor*[9] and the elucidation of the heterocaryotic life cycle in *Str. griseus*[1]. Though biochemical marker-traits were employed in these studies in order to check the genetic behavior as in any other works performed with other microorganisms, no detailed description in respect of the techniques for producing such mutants has been presented. Therefore it is convinced that the mutants are obtained rather easily through ultraviolet irradiation and the replica technique.

When the present study was first undertaken with a laboratory strain of *Streptomyces*, the frequency of mutant recovery was found to be quite low[8], but later, by adoption of the preincubation process preceding filtration and replica techniques, the frequency was significantly increased. The idea of concentrating biochemical mutants by letting wild type conidia germinate in a minimal medium and subsequently filtering them off through cloth or a glass filter has been adopted by many workers[2,10]. This technique was evidenced to be useful in the present case but the importance of the preceding preincubation process must be emphasized. Though it is still questionable whether or not the insert of the preincubation process may be invariably effective in producing biochemical mutants from any species of *Streptomyces*, the detail of mutant detection processes appearing in this paper will give some suggestions to mutation studies on *Streptomyces*.

MATERIALS AND METHODS

Microorganisms. A laboratory strain of *Streptomyces*, No. 142, identified as *Streptomyces griseoflavus* by gross morphological studies[8], was used as the original strain. This strain was chosen as a suitable material for the genetic study because its conidiospore formation was most distinct of all strains so far tested. Before this study, the strain was preliminarily purified several times by single colony isolation, and the pure culture was stocked on the modified Krainsky's medium in a refrigerator.

Media. Sporulation and stock media used were a modified Krainsky

type with the following composition: glucose 10 g., L-asparagine 0.5 g., K_2HPO_4 0.5 g., polypeptone 10 g., yeast extract 2.5 g., agar 30 g. and water 1,000 ml. The pH was adjusted to 7.2 with 5 N-NaOH before sterilization. The agar concentration was specially thickened to 3 % for the reason that it seemed to give favorable results on viability of the strain. The medium as well as media given below were sterilized at 120° for 5 minutes.

A complete medium consisted of : meat extract 10 g., polypeptone 10 g., yeast extract 2.0 g., glucose 7.5 g., NaCl 1 g., agar 15 to 20 g. and water 1,000 ml. (pH 7.2 with 5 N-NaOH).

A minimal medium employed here was one of a modified Czapek type consisting of : $NaNO_3$ 2 g., K_2HPO_4 1 g., $MgSO_4 \cdot 7H_2O$ 0.5 g., KCl 0.5 g., $FeSO_4 \cdot 7H_2O$ 0.018 g., glucose 30 g., purified agar[7] 15 g. and redistilled water 1,000 ml. For the preparation of solid medium of this type, glucose and the remaining components were dissolved separately in 300 ml. and 700 ml. distilled water respectively, and at the time when autoclaving at 120° for 5 min. was completed, both solutions were mixed together in a sterile flask. If the distribution of the medium from the big flask to smaller flasks was not entirely aseptic, the medium in the flasks were steamed again for 30 min. at 100°. In the process, special attention was paid lest that the medium should not be over-heated, since over-heating has unfavorable effects upon spore germination. The liquid medium used for the filtration process was prepared by autoclaving each of glucose, pottasium phosphate and the remaining components separately and by mixing them aseptically after cooling. This procedure was proved to be profitable for preparing such a medium containing less insoluble salts and that retains sufficient buffering action.

Supplemented media. For the growth of auxotrophic mutants, special kinds of media containing some of the following nutrients in 1,000 ml. of the minimal medium were employed: L-glycine 25 mg., L-histidine-HCl 25 mg., DL-methionine 100 mg., L-glutamic acid 200 mg., DL-tryptophan 150 mg., and each of the other amino acids 50 mg., each of nucleic bases 20 mg., each of vitamins 2 mg.

A limiting medium. For special purposes, the medium containing polypeptone 2 g. and yeast extract 0.4 g. in 1,000 ml. of the minimal medium was prepared.

Experimental procedures. Induction of mutation and isolation of auxotrophic mutants: Conidia were harvested from slant cultures on the sporulation or the complete medium after 7 to 10 day-incubation at 30°. Conidia in the range between 10^8 and 10^9 were suspended in 10 ml. sterile saline and placed in a Petri dish to expose them to uV-light. Irradiation were made with 15 watt Matsuda germicidal lamp at a distance of 40 cm. During the irradiation period, suspension was agitated gently. The irradiation period was adjusted from 2 to 4 minutes so that from 99 to 99.9 % of the irradiated conidia might be inactivated. After irradiation, the conidia were centrifuged and suspended again in 1 to 2 ml. of sterile saline, and each 0.4 ml. of this

suspension was spread over the surface of a sporulation or complete agar slant. After 7 to 10 day-incubation at 30° on these slants, the newly formed conidia were harvested into 10 to 20 ml. of sterile saline, sequentially centrifuged, washed twice with saline, and finally, about 10^9 of the conidia was inoculated into a reverse-*T* shaped tube containing 10 to 20 ml. minimal medium prepared as described above. The tubes were shaken gently for 15 to 20 hours at 30° on a Monod type shaker until slightly developed mycelia came to appear. After filtering off the mycelia through a Kaburagi glass filter, G3, No. 3, the filtrate containing conidia was transferred to a large sterile test tube to be kept at 30° in an incubator for several days. During this incubation, newly developed mycelia were filtered off each day through a sterile glass filter. In general, filtration was repeated 4 to 5 times to leave a suitable number of conidia in the suspension. After the treatments, each 0.2 ml. of the suspension containing about 50 ungerminated conidia was spread by a glass rod onto the complete agar medium in a Petri dish. The plates were incubated at 30° for one week.

The replica technique was carried out as follows[4] : the colonies developed on the plate were printed to minimal and complete agar plates by sterile velvet cloth placed on a cylinder made of wood, and after incubating the printed plates for 2 days at 30°, the type of colonies which could grow only on the complete medium but not on the minimal medium were selected to provide for further tests.

In parallel with these experiments described above, a kind of such comparative experiments, in which preincubation process prior to filtration was omitted, were carried out. In these cases, washed conidia were suspended in a minimal medium and irradiated with uV, and the suspension was transferred directly to reverse-*T* shaped tubes for the filtration process.

Determination of the growth factor requirement : the kind of colonies which grew on the complete medium but not on the minimal medium were purified further by single colony isolation, and requirement of growth factor was tested. Firstly, the Beijerink's auxanographic technique[6] was adopted: paper disks loaded with either casamino acid supplemented with tryptophan, nucleic base mixture, or vitamin mixture[3] were placed on a minimal agar plate on which more than 10^5 conidia of a mutant had been inoculated and agarlized. Most of the mutants responsed to casamino acid and further differentiation of their requirements were attempted by the use of paper disks loaded with each individual amino acid. Base or vitamin requiring mutants were tested in the same way. In many cases, the auxanographic technique did not give good results probably being due to the slow growth of the strain as compared with the fast diffusion of growth factors. For this reason, the auxanography was adopted for only gross grouping of the mutants.

Final checks of the auxotrophic mutants were made in the following way: twelve to sixteen mutants which were assumed to belong to the same

group were inoculated leaving a space around each of them on a complete agar plate. After several days, the colonies on the plate were printed by the replica technique onto minimal agar plates supplemented with or without

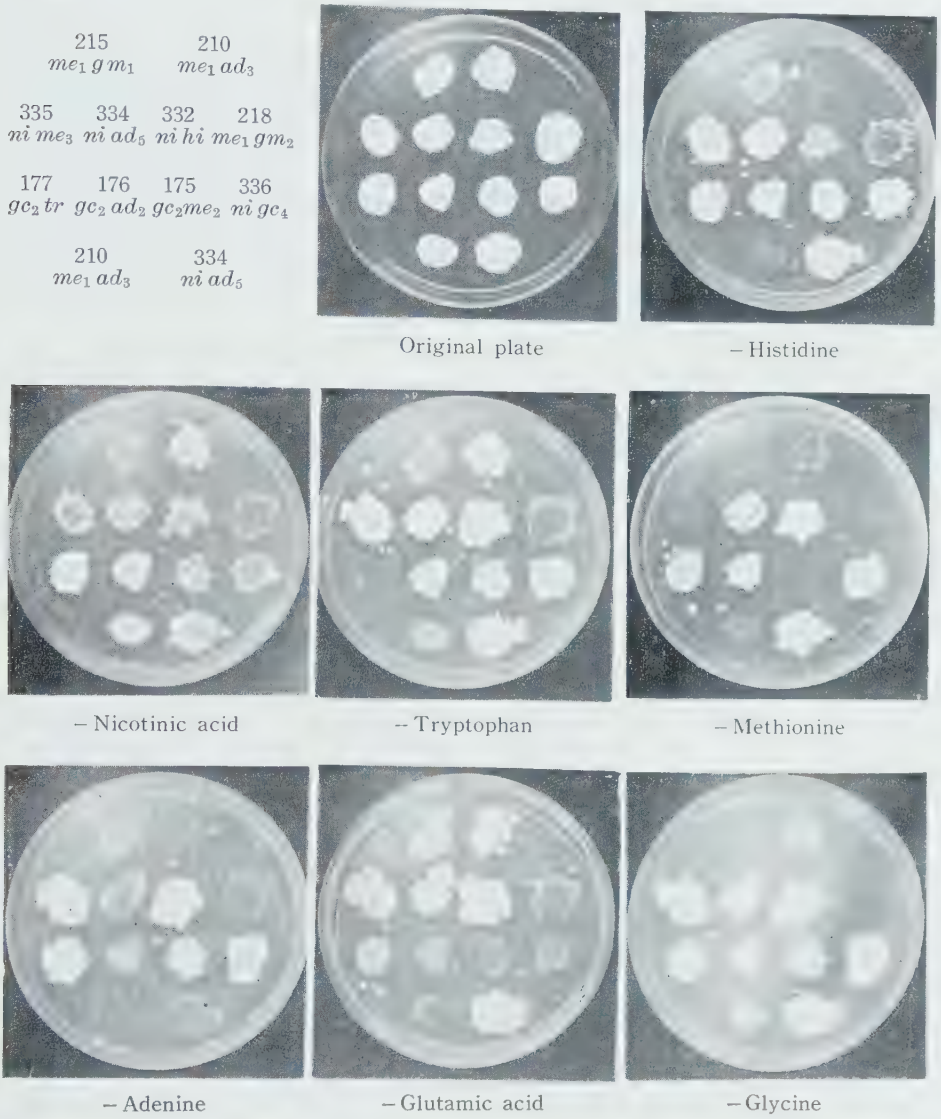

Fig. 1. Determination of Growth Factor Requirements by the Replica Method.
Basal medium: the minimal medium supplemented with histidine, nicotinic acid, tryptophan, methionine, adenine, glutamic acid, and glycine. Each plate is deficient in one of the growth factors.

growth factors, and both showed a growth response after 2 to 3 days, for example, to glutamate-supplemented minimal media and no response to a glutamate deficient medium was taken as evidence that the mutant required glutamate for growth (Fig. 1).

Syntrophism between the two mutants requiring the same growth factor was tested by inoculating them on limiting medium in a test tube. As a result, symbiotic growth, if existed, took place in the restricted area where the inoculation was made in double (Fig. 2).

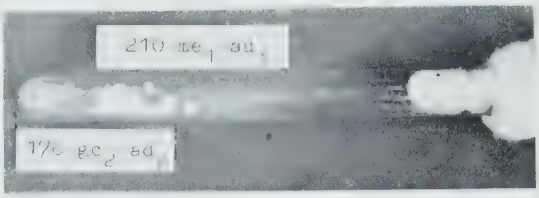

Fig. 2. Syntrophic Growth on the Limiting Medium between two Mutants Requiring the Same Growth Factor.

Growth factor requirement of a strain is to be expressed in this paper by a two-letter abbreviation of the corresponding growth factor. The same kinds of requirements are differentiated by suffix numbers according to their origins of isolation.

RESULTS

An attempt to isolate mutants from the wild type strain, No. 142, by uV-irradiation and the replica technique were reported previously[8]. At that time, the frequency of mutant recovery resulted considerably low as shown in Table 1, e.g., only two odd mutants, Nos. 261 and 305, were recovered

Table 1. Enhancement of Mutant Recovery by Insert of Preincubation Process.

Start-strain	Procedures for detection of mutants**	No. of mutants detected	No. of colonies examined	Frequency of mutant detection
No. 142 wild	uV ····· repl	1***	c. 50,000	2.0×10^{-5}
No. 261 *il**	uV ····· filt ··· repl	2	c. 2,500	8.0×10^{-4}
No. 305 *cy**	uV ····· filt ··· repl	3	c. 2,000	1.5×10^{-3}
No. 305 *cy**	uV ····· filt ··· repl	1	c. 1,000	1.0×10^{-3}
No. 142 wild	uV ··· incub ··· filt ··· repl	4****	430	9.3×10^{-3}
No. 21 *il** me_1	uV ··· incub ··· filt ··· repl	5****	400	1.3×10^{-2}
No. 33 *cy** ni	uV ··· incub ··· filt ··· repl	4****	700	5.7×10^{-3}
No. 17 gc_2	uV ··· incub ··· filt ··· repl	4****	1,500	2.7×10^{-3}

** uV: uV-irradiation, incub: preincubation on complete medium, filt: filtration process, repl: replica technique.

*** No. 261 *il** is omitted because of its ambiguous mutant character.

**** The same type of mutants which may have arisen from one mutational event is discounted.

from more than 50,000 colonies examined. Among them, No. 261 required isoleucine or leucine and No. 305 required cystine, cysteine, methionine, arginine, aspartric acid, glycine, phenyl alanine or nicotinic acid for growth, but strictly speaking, their requirement was not absolute, because it was observed that, especially in No. 261, they grew even on a minimal medium provided that inoculation was made heavily. Therefore, they are expressed with an asterisk as *il** or *cy**. Besides these, slow-growers and sterile mutants were obtained, but any attempt to lead them to full growth by supplementing nutrients resulted in failure.

In the next step, both the filtration technique and the replica technique were adopted to produce double auxotrophic mutants from these odd mutants. In place of the minimal medium, isoleucine or cysteine supplemented media were employed in these experiments. As a result, several auxotrophs were recovered after a series of trials, indicating that the application of the filtration process together with the replica technique was more profitable for the isolation of mutants than the single use of the replica technique. Strains, No. 21 (derived from No. 261), Nos. 32 and 33 (both from No. 305) required methionine, arginine and nicotinic acid respectively. On the minimal medium supplemented with one of these nutrients, the corresponding mutant grew well even if the medium was deficient in isoleucine or cysteine. Therefore these mutants were sometimes described as auxotrophic mutants having only one requirement, in neglect of the *il** or *cy** character. Because the mutant character of the strain, No. 32 ar_2, reversed in a fairly high frequency, the remaining two mutants were used for the following experiments.

When the irradiated conidia of strain, No. 21 me_1 or No. 33 *ni* were preincubated on the complete medium so as to pass through one life-cycle before the filtration and the replica techniques, the frequency of mutant recovery was remarkably improved (Table 1). When several mutants of the same type which might have arisen from the same origin were recovered in one attempt, they were counted as one mutant. It was also proved that this series of procedures was effective in producing mutants from the wild type strain No. 142. A strain, No. 17, which required glycine or serine for growth was thus obtained. The double (or triple) mutant strains listed in Table 2 were derived from either strain No. 17 gc_2, 21 me_1 or 33 *ni* via the method just mentioned.

Besides the mutants described above, a considerable number of mutants requiring some unknown growth factors were obtained, that is, they did not grow on any media supplemented with ready known growth factors. Some of them seemed to have requirements of peptide-like compound because these mutants grew on casamino acid-supplemented media but failed to grow on media containing amino acid mixture. As shown in Table 2, some of the determined mutants require alternative growth factors such as glycine or serine, aspartic or glutamic acid, and adenine, xanthine, hypoxanthine or

uric acid, but the characters are expressed by the representative one.

At the same killing rate and in the similar procedures as in the case of uV-irradiation, X-ray irradiation (200 KV. 20 mA.) produced no auxotrophic

Table 2. Mutant Characters Obtained by Present Procedures

Requirement	Strain carrying the character**	Alternative requirement and comments
Arginine	12 ar_1 32 *cy* ar_2	Glutamic or asparatic acid for ar_1
Cystine	305 *cy**	Cysteine, methionine, arginine, aspartic acid, glycine, phenylalanine or nicotinic acid.
Glutamic acid	215 *il** me_1 gm_1 (white) 218 *il** me_2 gm_2 (pink)	Aspartic acid for gm_1
Glycine	16 gc_1 (white) 17 gc_2 (pink) 214 *il** me_1 gc_3 336 *cy** *ni* gc_4	Serine for every character; $gc_2 \fallingdotseq gc_3$***
Histidine	332 *cy** *ni* *hi*	
Isoleucine	261 *il**	Leucine; slower growth
Methionine	21 *il** me_1 175 ge_2 me_2 335 *cy** *ni* me_3	
Tryptophan	177 gc_2 *tr*	
Casamino acid	171 gc_2 ca_1 213 *il** me_1 ca_2	Responds to casamino acid but not to amino acid mixture for ca_1 and ca_2
Adenine	22 *il** ad_1 176 gc_2 ad_2 210 *il** me_1 ad_3 212 *il** me_1 ad_4 334 *cy** *ni* ad_5	Xanthine, hypoxanthine or uric acid; spared by histidine for ad_3 and ad_5; $ad_2 \fallingdotseq ad_3$***
Nicotinic acid	33 *cy** *ni*	
Vitamins ?	34 *cy** *vi*	Responds slightly to vitamin mixture

** Suffix number is added according to its origin of isolation. Difference in color of vegetative growth is indicated in parentheses.

*** The mark, $\fallingdotseq$, indicates possible unidentity of characters according to the syntrophism test.

mutant among about 3,000 colonies examined. However, it is interesting of note that the survival curves were evidently of a "two-hit" type, which will be reported elsewhere in detail.

There were several mutants which required the same kind of growth factor though they had been obtained independently. For example, both strains, Nos. 176 gc_2 ad_2 and 210 me_1 ad_3, required adenine for growth. When the conidia of these mutants were streaked together on a limiting medium supplemented with sufficient amounts of both glycine and methionine, vigorous growth was observed after 3 to 5 day-incubation in the area where both mutants had been inoculated mixedly. Such growth might be due to the syntrophism or the heterocaryosis between the two strains (Fig. 2), and it might be suggested that their requirements for adenine were based on the different genetic and biochemical activities. The possible unidentity of the two mutant characters is noted in Table 2, only in the case where the remarkable syntrophic or heterocaryotic growth was observed.

DISCUSSION

Bradley and Lederberg have reported that the frequency of auxotrophic mutants among uV-irradiated conidia in *Streptomyces griseus* was around 0.1 %[1], and Sermonti and Spada-Sermonti have also found that the frequency in *Str. coelicolor* was from 0.05 to 1 % of the uV-irradiated conidia[9]. In contrast to these data, the frequency of mutant detection in the present study was far less than 0.01 %, though it is assumed that a similar technique has been employed throughout these three works. Moreover, this frequency is still quite small even as compared with many reports dealing with the random isolation of auxotrophic mutants in various microorganisms.

The filtration technique raised the frequency to the order of 10^{-3} to 10^{-4}, but there still existed some difficulties for extending this technique to *Streptomyces*. The main difficulty encountered was that, during the course of incubating long enough to concentrate mutants through the filtration process, a considerable number of mutants as well as of wild type conidia has lost their viability. In order to minimize the inactivation of conidia, it is preferable to call attention to the following two points. First, as the buffering action of the minimal medium intended for use of the filtration process is very much influential on the viability of conidia, the medium must be carefully prepared as described previously. Second, the cultivation-period on a shaking machine must be shortened as far as the homogeneous germination of conidia is possible. According to the preliminary test of viability, an amount exceeding 90 % of conidia was inactivated within a few days, provided they were shaken in a medium which did not permit germination.

When the irradiated conidia were preincubated on the complete medium before filtration, the frequency of mutant detection was further improved.

This effect (termed preincubation effect) might be due to the following reasons: (1) the growth of auxotrophic mutants on the complete medium was faster than that of the wild type, so that the mutants might be selected during preincubation. However this assumption is improbable because, as a matter of fact, in many cases the growth of recovered mutants was not so fast as that of the wild type. (2) In the case where the preincubation process was inserted before the filtration, about 10^8 conidia were usually inoculated into one ml. of minimal medium, while in the case where no preincubation was made, only 10^6 to 10^7 survived conidia were inoculated. Therefore, if the frequencies of mutants were equal in both cases, the absolute numbers of mutants in an equal volume of the conidial suspension, and consequently, the mutant conidia which would be left behind through the filtration, might be more abundant in the former case than in the latter, thus resulting in the preincubation effect. Since, however, even when the conidium density in either case had been modified by dilution or centrifugation so as to result in a similar concentration as in each other, the preincubation effect was still revealed, therefore, the conidium density in the minimal medium might not be the main cause of the effect. (3) The preincubation effect might be provoked by the delayed appearance of mutation in uV-irradiated conidia, possibly through the mechanisms described below: First, the conidia containing affected nuclei might germinate developing mycelia and be filtered off before being stabilized as mutants, and second, the conidia containing both wild and mutant nuclei might form heterogeneous colonies on the complete medium, thus the detection of mutant segregants by the replica technique was prevented. These possibilities will be discussed below.

In the study of radiation-induced mutation of bacteria, the delayed appearance of mutants was interpreted as results of the following effects[5]. (1) Segregation delay; if there are more than one nucleus (or gene complement) in a cell and, if only one of them is affected by the mutagen, the mutant character will appear after several cell divisions and after consequent nuclear (or gene) segregation. (2) Phenotypic delay; a mutant gene may require a period of metabolic activity before it is able to change the phenotype. (3) Mutation delay; a mutagen may not act directly on a gene, so that there may be a time-lag between the treatment and the occurrence of real gene mutation. (4) Irregularity delay; the irregular onset of division after treatment may possibly be counted as a cause of the delayed appearance, because growth of the affected cells is more delayed than the unaffected cells.

Among the four mechanisms showing the delayed appearance of mutation, neither phenotypic delay nor irregularity delay may cause the loss of mutants through filtration and neither may affect mutant detection by the replica technique. Therefore, these two mechanisms are unaceptable as possible working mechanisms for the preincubation effect. Among the remaining two mechanisms, i.e., segregation delay and mutation delay, of which either

may possibly be a cause of the preincubation effect, the latter seems unfavorable for explaining the difficulty in auxotroph detection among conidia plated directly after uV-irradiation. Moreover, as referred briefly in the experimental results, X-ray inactivation gave a survival curve of a "two-hit" type, suggesting the two sets of genetic units in a conidium. As a consequence, the segregation delay seems to be the most preferable assumption to give an explanation of the present situation.

Anyhow, it may be suggested that the delayed appearance of mutation must be taken into account when such strains as *Str. griseoflavus*, in which the chance of mutant detection is not so frequent, are dealt with. Moreover, as this type of delayed appearance may be encountered so frequently, even in the improvement of antibiotic production by means of induced mutation of *Streptomyces spp.*, the profit of adopting the preincubation process in such cases must be emphasized.

SUMMARY

Auxotrophic mutants of *Streptomyces griseoflavus* have been isolated by the following method. After irradiating conidiospores with ultraviolet light, the survivors, equivalent to 0.1 to 1 % of the conidia irradiated, were incubated on complete agar for a week and, to the newly arrived conidia, filtration technique was applied in order to concentrate the growth factor-requiring mutants. They were then recovered among the colonies on a complete medium with the aid of the replica plating method. Preincubation prior to filtration proved important, because the frequency of mutants recovered among colonies on the complete medium was raised from 0.1 to 1 % by this process. The mutants required the following growth factors: methionine, arginine, cystine, tryptophan, nicotinic acid, glycine or serine, adenine, xanthine, hypoxanthine or uric acid, and glutamic acid or aspartic acid.

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RADIOKINETIC EVIDENCES FOR THE BIPARTITE OR DIPLOID NUCLEUS IN CONIDIA OF *STREPTOMYCES GRISEOFLAVUS*

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In the course of work attempted for isolating auxotrophic mutants of *Streptomyces griseoflavus*, it was found that the frequency of mutant recovery was significantly increased by incubating conidia on a complete medium after ultraviolet treatment, that is, when uV-irradiated conidia were pre-incubated on the complete medium for a week before letting the newly-formed conidia stand in a minimal medium for the filtration process, about 1% of the conidia existing in the filtrate was auxotrophic, while the frequency was approximately 0.1% when tested without preincubation. Though the mechanism of preincubation effect was unclarified, the authors referred to the notion that this strain might contain two sets of genetic units in a conidium and that the so-called segregation delay might be the most possible mechanism[6].

When the conidia were irradiated by X-rays instead of ultraviolet light in the expectation of high frequency of induced mutation, survival curves having a shoulder in the early period of irradiation and resembling to "two-hit" curves have resulted. In the present paper, further analysis of X-ray inactivation will be presented as another evidence for supporting the view that the nucleus in a conidium of *Str. griseoflavus* has replication of genetic units. It is likely that the nucleus is bipartite or diploid, because, according to the cytological studies that are to be published elsewhere, the conidium is apparently uninuclear[7].

MATERIALS AND METHODS

Microorganisms. A laboratory strain of *Streptomyces griseoflavus*, No. 142, and the two induced mutants, No. 21 dependent of isoleucine and methionine and No. 305 dependent of cystine, were employed for this study. Details of the history and characters of these strains were reported elsewhere[6][8]. Besides these, three species of Streptmyces fungi, *Str. griseus* Waksman No. 1, *Str. kitasatoensis*, and *Str. coelicolor* 1^+ (available through the courtesy of Dr. G. Sermonti) were used for the comparative study.

Media. Sporulation and stock media used for *Str. griseoflavus* and *Str. griseus* were of a modified Krainsky type supplemented with yeast extract

and peptone. For colony counting, bouillon-glucose medium was employed. The conidia of *Str. coelicolor* were harvested from the culture on a Czapek type medium. The composition of these media were the same as described previously[6].

The medium used for the sporulation of *Str. kitasatoensis* was as follows: glucose 5 g., starch 25 g., soybean meal 20 g., dry yeast 5 g., urea 0.5 g, $(NH_4)_2SO_4$ 5 g., NaCl 5 g., K_2HPO_4 1 g., $CaCO_3$ 3 g., agar 1.8 g., and water 1,000 ml. (pH 7.2 with 5 N-NaOH).

Experimental procedures. Conidia harvested from 6 to 10 day-culture at 30° (in some cases older cultures were used) on an appropriate medium were suspended in sterile saline, and vibrated vigorously for 20 min. by an electric vibrator. Large particles were removed by filtrating the suspension through a Kaburagi glass filter G 3, No. 3. By these treatments, the frequency of single conidium in the suspension exceeded 90% of the total count. The remainder consisted of aggregates of conidia and minute hyphal fragments.

The rate of viable conidia to total conidia in the suspension deviated from 10 to 90% according to the strain and the culture-period. Ten ml. of the suspension containing about 10^6 total conidia per ml. was transferred to a sterile beaker covered with a glass plate, diameter one inch, and was exposed to X-rays at a distance of 18 cm. from the target of a Matsuda KXC-18 type X-ray projector which was operated at 200 Kvp, 20 mA. The X-rays were filtered through a 1.0 mm. alminum plate and intensity at that distance was 1,000 r per min. The beaker containing suspention was rotated gently so as the whole area could get a constant dose during irradiation. The irradiation was made for successive 12 min. intervals for the low dosages, and 24 min. for high dosages. After different times of exposure, 0.1 ml. of the sample was removed, diluted with sterile saline and plated in triplicate on complete agar. The samples without exposure were taken from the original solution as the control and diluted independently in two systems so that the count might not be affected by dilution error. The survival number after irradiation was determined by counting the average number of colonies appearing after 4 to 5 days at 30°. Such a long incubation was necessary since in some of the irradiated conidia, germination started at a later period. Only the plates on which 20 to 300 colonies developed were counted and registered, because the number of colonies appearing was proportional to the dilution rate only in this range.

RESULTS

When the conidia of wild type strain, No. 142, of *Str. griseoflavus* were irradiated by X-rays, the dose-survival relationship was not exponential but rather sigmoidal, i.e., the surviving curve in a logarithmic scale plotted against the X-ray dose had a clear shoulder, and the extrapolation of the

straight portion intercepted the ordinate at 2.3, as shown in Figure 1. This fact was confirmed by three independent experiments. The first data were obtained by the use of conidia harvested from 6 day-culture on the sporulation medium (the viability of conidia was 90%) and the second from 25

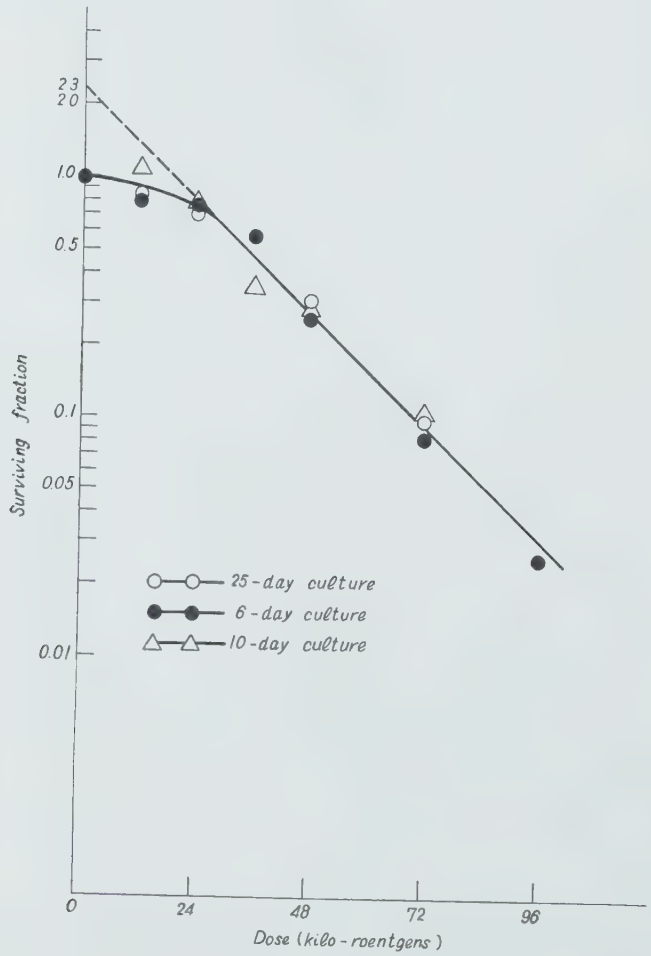

Fig. 1. Survival after X-irradiation of *Str. griseoflavus*.

day-culture (viability: 42%). In the first case, the suspension with less than 2% clumping conidia were specially prepared by filtrating the conidia suspension three times. As compared with the first two, the conditions employed for third data were considerably different; the conidia were harvested from 10 day-culture, of which the viability was incidentally low (12.5%), and suspended in saline resulting in a solution containing 10^6 total conidia per ml. This suspension was irradiated by X-rays at 5°, much lower than the

temperature (20–25°) at which the former two were treated. As these three data coincided well as seen in Figure 1, it appears that the survival curve is independent of the environmental conditions and is controlled by the intrinsic character of the strain.

When two auxotrophic mutants, No. 21 *il** me_1 and No. 305 *cy**, both of which were induced from the strain No. 142 by uV-irradiation, were treated with X-rays, the survival curves shown in Figure 2 were obtained (viabilities

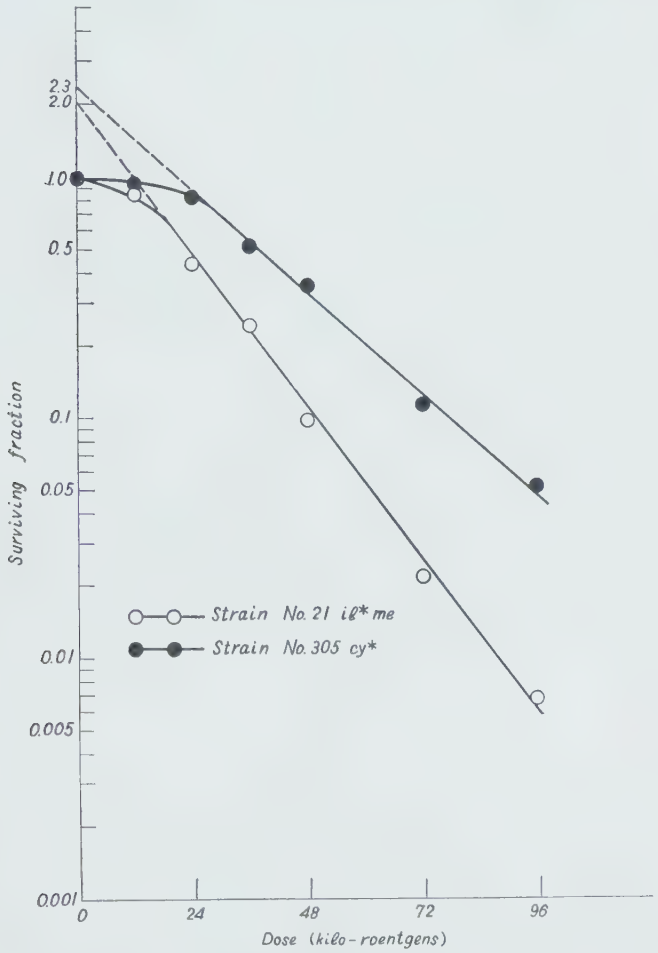

Fig. 2. Survival after X-irradiation of Auxotrophic Mutants of *Str. griseoflavus*.

of conidia: 79% and 50%, respectively). Though the length of shoulders and the slopes of the straight portion were somewhat different from that of the strain No. 142, they were still sigmoidal and extrapolated to approximately 2 at the zero dose. Therefore, such a constant extrapolation value

may not be caused by the reflection of experimental conditions, but rather by the genetic structure of the strain.

In order to make a comparison with *Str. griseoflavus*, three species of *Streptomyces*, *Str. griseus*, *Str. coelicolor* and *St. kitasatoensis*, served for the radiokinetic analysis. In the latter cases, the conidia were harvested from cultures on the Czapek's medium or on the sporulation medium specially prepared, because the strains did not form sufficient amount of conidia on the modified Krainsky's medium. By exposing the conidia of these three strains to X-rays in the same conditions as described above, three survival curves shown in Figure 3-I, II and III, were obtained. Though the curves

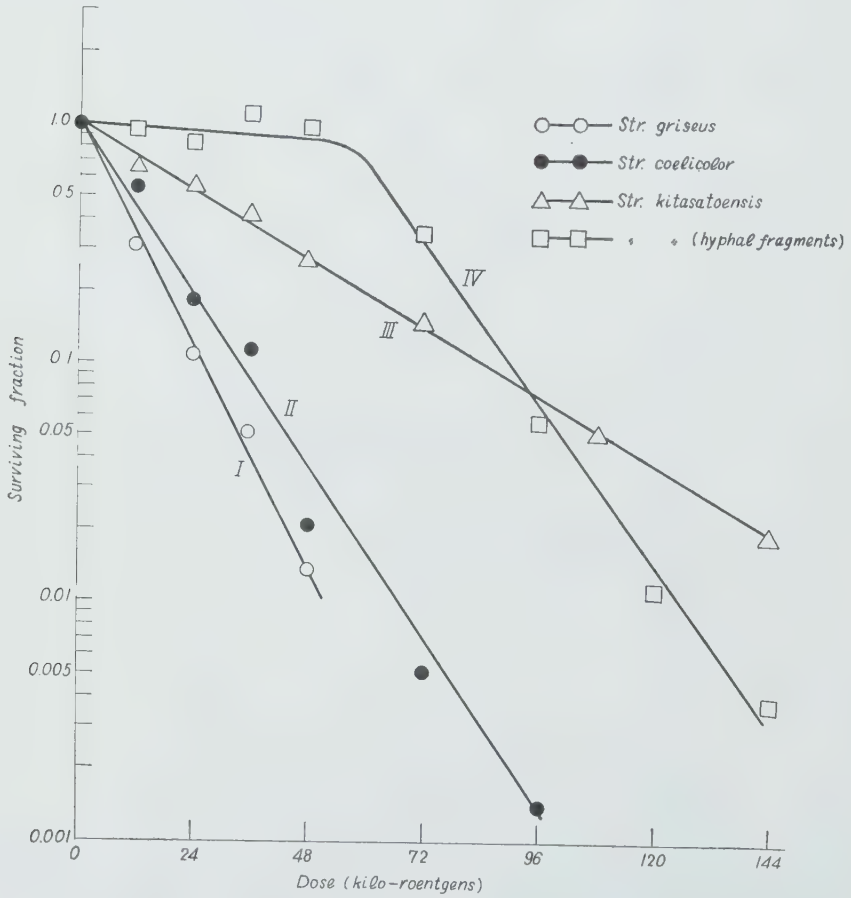

Fig. 3. Survival after X-irradiation of *Str. griseus*, *Str. coelicolor*, and *Str. kitasatoensis*.

have different slopes from that of *Str. griseoflavus*, they are apparently exponential. These results are consistent with the accepted belief that most

species of *Steptomyces* as well as other microorganisms give exponential survival curves when irradiated by X-rays, and, furthermore, suggest that the sigmoidal survival curve of *Str. griseoflavus* may not be due to any technical error in X-ray irradiation process, but to characteristic biological behavior.

In the next experiment, X-ray inactivation test of hyphae, which was enough in length to contain several nuclei in each fragment, was attempted in the expectation of obtaining a survival curve having a shoulder if the type of curve might depend on the number of hereditary units such as nucleus in each cell. *Str. kitasatoensis* was used for this purpose and hyphal suspension was prepared in the following way. Many hyphal fragments were scraped off from the 10 day-culture on the modified Krainsky's medium and suspended into sterile saline; this suspension contained few conidia, since the strain hardly conidiated on this medium. Clumps of hyphae were cut into small pieces by stirring the suspension vigorously with a homogenizer. Then the hyphal fragments which were free of conidia were collected by centrifuging at a slow speed. Long fragments and remained aggregates were filtered off through a glass filter, G 3, No. 2 (not by No. 3), so that only the hyphal fragments with the length to 10 to 50 μ remained in the suspension. Thus the prepared suspension was exposed to X-rays in the same way, as in the case of conidial suspension. Figure 3-IV shows one of the survival curves obtained with the hyphal fragments; it has a long shoulder as was expected. Repetition of this kind of experiment, revealed that the lengths of the shoulder were variable according to the lengths of hyphae employed. In general, however, the survival curves extrapolated to around 10, and this value was consistent with the average number of chromatinic substances contained in the hyphae having the length of 10 to 50 μ.

Besides the hyphal fragments harvested from solid culture as described above, the hyphae prepared by the shaking culture resulted in a similar X-ray inactivation curve.

DISCUSSION

During the past decades, Lea's target theory has been widely accepted in the field of radiation biology, and it has been known that the conidia of *Streptomyces* spp. as well as many kinds of microorganisms give exponential survival curves as typical results upon inactivation by ionizing irradiation. According to Lea[2], the occasional finding of a sigmoidal curve in the radiokinetic study of microorganisms may be due to some disturbing factors. One possible disturbing factor may be the presence of aggregates of the cells in the suspension, but this factor might not act in the present studies, since more than 90% of conidia were exactly single in every preparation. A second disturbing factor suggestible may be the accumulation of certain toxic substances which are produced by the irradiation and may secondarily

act upon the microorganisms. In case of small dosages, the concentrations of the poisons, if toxicity exists, would be insufficient to kill any cell, and the rate of death observed would be due to the direct action of radiation on the cells. With the increase of dosage the concentrations of the poisons become rich enough to cause appreciable mortality, and death will be accelerated by both the direct action of radiation and the secondary action caused by the poisons; thus an apparently sigmoidal curve may be resulted. In the present study, however, the conidial suspension irradiated by X-rays was considerably diluted before being plated, so that the toxic substances, if any, may not affect the germination of conidia, and besides, it is unlikely that the poisons selectively acted toxically upon *Str. griseoflavus* without affecting any other strains which gave exponential survival curves.

Moreover, it is not probable that the sigmoidal survival curves were produced by any accidental errors, but, on the contrary, according to the following reasons, it is considered that the curves were due to the intrinsic character of the strain. One evidence is that survival curves were highly reproducible independently of enviromental conditions, either of the culture or of the irradiation. Second, X-ray inactivation of mutant strains derived from *Str. griseoflavus* revealed the same type of survival curves, and were invariably extrapolated around 2 in spite of the fact that slopes of the straight portion differed so much among the wild strain and the auxotrophic mutants. As the next evidence, several other species of *Streptomyces* tested in the same way evidently gave exponential survival curves. This fact is consistent with the notion that most *Streptomyces* have a single target in one conidium, as pointed out above. As an additional study indicating the interrelation between the target theory and the biological background, it was proved that the inactivation curve of hyphal fragments was a sigmoidal one which gave the same extrapolation value as the observed number of nuclei in one fragment. For the above-mentioned reasons, the apparent "two-hit" curves obtained by irradiating the conidia of *Str. griseoflavus* would reflect the presence of double targets[1], or probably double genetic units, in one conidium.

Employing a family of polyploid yeasts, Latarjet and Ephrussi, and later Lucke and Saracheck suggested that a single target sensitive to X-rays existed for each chromosome set[3,4]. According to the radiokinetic study of yeasts by Pomper *et al*[5], some diploid strains showed exponetial curves, nevertheless they described that strains which gave "two-hit" type curves were diploid.

With reference to these preceding studies, it may be said that the conidia of *Str. griseoflavus* which give "two-hit" type survival curves involves double genetic units in each cell. Under this assumption, the segregation delay which was experienced in the auxotroph detection described in the previous report is readily apprehended. In this connection, it must also be commented that the cytological study of the strain reported elsewhere has

already presented another evidence that the conidia are uninuclear and that the nucleus develops from a "dumpbell" shaped chromatinic substance which might consist of two units. For this reason, it is the conclusion of the present studies that the conidium of *Str. griseoflavus* may comprise a bipartite or diploid nucleus in each cell.

SUMMARY

In contrast to several species of *Streptomyces* which revealed exponential survival curves by X-ray inactivation tests, *Str. griseoflavus* and auxotrophic mutants of the strain gave apparently "two-hit" curves. On the ground of the interrelation between radiokinetic and biological findings, a proposal that the conidium of *Str. griseoflavus* comprises a bipartite or diploid nucleus in each cell was presented.

ACKNOWLEDGEMENTS

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STUDIES ON THE PENTOSE METABOLISM BY MICROORGANISMS

1. A NEW TYPE-LACTIC ACID FERMENTATION OF PENTOSES BY LACTIC ACID BACTERIA

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The fermentation of various pentoses by lactic acid bacteria was first described by Fred, Peterson and Anderson[1], and the following formula was proposed for the reaction:

$$C_5H_{10}O_5 \longrightarrow CH_3CHOHCOOH + CH_3COOH \qquad (I)$$

This formula has, heretofore, been admitted by many investigators. According to the formula equimolar quantities of lactic and acetic acids are formed from pentose, namely, the theoretical yield of lactic acid is 60% by weight of the consumed pentose. By an isotopic experiment using *Lactobacillus* sp.[2],[3],[4] it has been established that the methyl and carboxyl groups of acetate arise from C-1 and C-2, while -COOH, -CHOH and $-CH_3$ groups of lactate are derived from C-3, C-4 and C-5 of pentose molecule respectively.

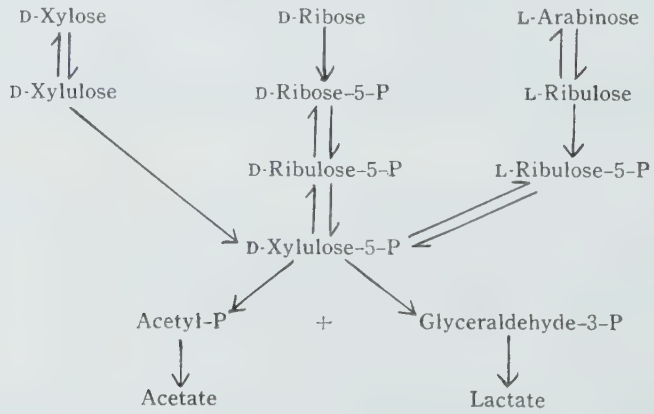

Fig. 1. Pathway of Pentose Fermentation by *Lactobacillus pentosus*.

Recently, the pathway of pentose fermentation by *L. pentosus* has been summerized by Horecker and his co-workers[5] as shown in Fig. 1. In this

scheme each pentose phosphate ester is converted to D-xylulose-5-phosphate which is then cleaved by phosphoketolase into glyceraldehyde-3-phosphate and acetyl-phosphate. These resulting compounds are further converted to lactate and acetate respectively. By a comparative study on the fermentation of pentoses, however, we have observed the following two cases[6]: 1) some strains of lactic acid organisms metabolize various pentoses according to formula (I), and 2) the others produce lactic acid in a yield of more than 60% of consumed pentoses with a small amount of formate, but no detectable amount of acetic acid. For example, *Lactobacillus arabinosus* readily ferments L-arabinose and produces equimolar quantities of lactic and acetic acids as expected from formula (I), while on the other hand *Streptococcus faecalis* produces 1.45 moles of lactate and 0.23 mole of formate per mol. of L-arabinose without any detectable amount of acetate[6]. Furthermore, the cells of *S. faecalis* grown in a medium containing L-arabinose is able to produce lactic acid in a yield of 95% of the consumed L-arabinose (1.58 moles of lactate per mole of L-arabinose consumed), while volatile acids are not detectable. This finding gives a clear evidence that *S. faecalis* might involve a new pathway for pentose fermentation. We have much interests in those phenomena mentioned above, especially in the new pathway of pentose fermentation.

In this paper, it will be discussed that this new type of pentose fermentation may be formulated as follows:

$$6\ C_5H_{10}O_5 \longrightarrow 10\ CH_3CHOHCOOH \qquad (II)$$

METHODS

Strains and medium—Lactic acid bacteria used in the present experiments have been maintained as stab-culture on a nutrient bouillon-malt extract-$CaCO_3$ agar by transferring at 3 month-interval in our laboratory. The medium for seed culture and fermentation has the following composition: peptone 1 g; fish meet extract 1 g; NaCl 0.5 g; Difco's yeast extract 0.2 g; pentose 2–3 g; $CaCO_3$ 3 g; and tap water 100 ml. Sterilization was carried out as mildly as possible under a pressure of 10 lbs. for 1 min. in an autoclave in order to minimize the possible decomposition of pentoses into formate at a high temperature. Seed cultures were prepared by inoculating organisms from stock cultures and incubating them at 30° or 45° for 24 hours. The fermentations were generally carried out for 5 days at 30° after inoculating 5 ml of seed culture of each strain into 200 ml fermentation medium, except for *Lactobacillus thermophilus* which is cultured at 45°C.

Determination of Volatile Acids[7]—The cultures were centrifuged at 4,000 G for 10 minutes then the supernatant was acidified with sulfuric acid. Volatile acids were separated from the acidified broth by steam-distillation and identified paper-chromatographically. The quantitative determination was carried out by the fraction distillation method[8].

Determination of Lactic Acid—After removal of volatile acids, the acidified broth was extracted with ether for 24 hours. Acids in the ether extract were estimated as lactic acid by two methods, i.e., alkali titration and colorimetry[9]. In fact, the nonvolatile acid contained in the ether extract was identified exclusively to be lactic acid by paperchromatography.

Determination of sugars—For the determination of pentoses the Bertrand method was applied, and for the identification and determination of ketoses the cystein-carbazol reaction[10], $FeCl_3$-orcinol reaction[11], and paperchromatography[12] were employed.

Preparation of the cells for small-scale fermentation—The cells were prepared as follows: *S. faecalis* was grown in a medium containing L-arabinose and *L. thermophilus* T_1 in D-xylose medium. After 20 hours culture, the cells were harvested by centrifugation and washed three times with distilled water. About 2 g of wet cells of both organisms were obtained from each one liter of the culture broth.

Measurement of radioactivity—Radioactivities of all produts were determined after conversion to salts or barium carbonate.

RESULTS

1) *Fermentation of pentoses by various strains of lactic acid bacteria*

From the results of fermentation shown in Table 1, types of pentose fermentation by lactic organisms were divided into following two types: Type 1) which produced equimolar lactic acid and acetic acid from pentose according to formula (I) and Type 2) which produced more than one mole of lactate and small amount of formate but no acetate from one mole of the substrate. The latter type of fermentation may be formulated as follows:

$$C_5H_{10}O_5 \longrightarrow \text{more than one mole of } CH_3CHOHCOOH + x\ HCOOH \quad \text{(III)}$$

In these experiments it was observed that some strains were able to metabolize pentoses according to formula (I) and (III). For example, *Lactobacillus thermophilus* II was able to ferment L-arabinose as formula (I) and D-xylose as formula (III). These phenomena suggest the fact that the pentose metabolizing systems of lactic organisms are sometimes altered by natures of pentose used in the culture media. Further, as shown in Table 2, *L. xylosus* was able to ferment D-xylose and D-ribose through different metabolic systems. On the other hand, *L. thermophilus* T_1 metabolized various pentoses according only to formula (III) and *Leuconostoc mesenteroides* B07 followed exclusively to formula (I).

2) *Effect of carbonate on the pentose metabolism by S. faecalis and L. thermophilus* T_1

When a $CaCO_3$-containing medium is employed, the broth may be maintained nearly saturated conditions with CO_2 and HCO_3^- throughout the fer-

Table 1. Fermentation of Pentoses by Lactic Acid Organisms.

a) Fermentation of L-arabinose

Strain	Initial sugar (g)	Consumed sugar (g)	Latic acid (g)	Acetic acid (g)	Formic acid (g)	Yield of lactic acid (%)
S. faecalis	3.75	3.60	3.11	0	0.19	86.4
S. glycerinaceus	3.75	3.75	3.11	0	0.24	82.9
L. arabinosus	3.75	3.75	1.98	1.56	0	52.8
L. thermophilus T_1	2.45	2.20	1.55	0	0.24	70.4
L. thermophilus II	2.80	1.93	1.18	0.52	0	61.2
Leuc. mesent. B07	3.75	3.20	1.76	1.33	0	55.0
L. brevis	3.75	2.35	1.13	1.04	0	48.0
*Pc. hennebergi**	2.05	1.78	0.95	0.41	0	53.3
*Pc. lindneri**	2.05	1.40	0.71	0.60	0	50.7

* 100 ml culture.

b) Fermentation of D-xylose

S. lactis	3.30	3.30	2.01	1.02	0	60.9
L. xylosus	4.12	3.06	1.63	1.10	0	53.2
L. thermophilus T_1	3.24	2.85	2.34	0	0.29	82.1
L. thermophilus II	2.45	1.45	0.98	0	0.41	67.5
Leuc. mesent. B07*	14.2	8.5	4.52	2.88	0	53.1
L. fermentum H60	2.80	2.65	1.35	1.47	0	50.9
Pc. hennebergi	2.10	1.99	0.95	0.41	0	47.8
Pc. lindneri	2.10	1.72	0.84	0.80	0	48.9

* 700 ml culture.

c) Fermentation of D-ribose

S. faecalis	0.93	0.83	0.68	0	0.04	81.9
S. lactis	0.50	0.50	0.32	0	0.07	64.0
L. xylosus	0.80	0.67	0.50	0.03	0.07	74.6
L. arabinosus	0.50	0.50	0.22	0.13	0	44.0
L. thermophilus T_1	0.93	0.93	0.66	0	0.04	70.9

50 ml culture.

mentation, because they are liberated from carbonate by organic acids formation from sugars. Therefore, two attempts were made in order to determine whether the carbonate fixation reaction was involved or not in this fermentation, namely 1) comparison of fermentations in the presence and absence of carbonate, and 2) investigation of the incorporation of C^{14}-carbon into lactate by using $NaHC^{14}O_3$.

Table 2. Pentose Fermentation Formula by Lactic Acid Organisms.

Strain \ Substrate	L-Arabinose	D-Xylose	D-Ribose	D-Glucose
S. faecalis	III	non-ferment.	III	homo
S. glycerinaceus	III	non-ferment.	——	homo
S. lactis	non-ferment.	I	III′	homo
L. xylosus	non-ferment.	I	III′	homo
L. arabinosus	I	non-ferment.	I	homo
L. thermophilus T_1	III	III	III	homo′
L. thermophilus II	I	III	non-ferment.	homo′
L. brevis	I	non-ferment.	——	hetero
L. fermentum H60	non-ferment.	I	——	hetero
Leuc. mesent. B07	I	I	non-ferment.	hetero
Pc. hennebergi	I	I	——	homo
Pc. lintneri	I	I	——	homo
*L. pentosus**	I	I	——	——

* by Horecker et al.
non-ferment., non fermentable.; ——, not examined.; III′, almost III.

1) In the absence of carbonate, lactic acid fermentation was proceeded by neutralizing the broth with freshly prepared N-NaOH at a 12-hours interval. As is shown in Table 3, *S. faecalis* produced 1.35 moles of lactate from one mole of L-arabinose and *L. thermophilus* T_1 produced 1.22 moles of lactate from one mole of D-xylose, and at the same time small amounts of formate were formed by both strains. These results are similar to those of fermentations obtained by using Ca-carbonate containing medium (see Table 1), and suggests that carbonate has no effect on the pathways of pentose metabolism by *S. faecalis* and *L. thermophilus* T_1.

2) In the isotopic experiments for the carbonate fixation, $NaHC^{14}O_3$ was used. One half of the $NaHC^{14}O_3$ used in this case was added at the initial

Table 3. Fermentation of Pentoses in the Absence of Carbonate.

Strain	*S. faecalis*	*L. thermophilus* T_1
Substrate	L-Arabinose	D-Xylose
Initial sugar (g)	1.80	3.05
Consumed sugar (g)	1.68	1.05
Lactic acid (g)	1.37	0.77
Formic acid (g)	0.11	0.18
Carbon dioxide (g)	trace	trace
Yield of lactic acid (%)	81.6	73.3
Do. mol./mol.	1.35	1.22

mol./mol.: moles of produced lactate per moles of consumed sugar.

stage of fermentation process and another half at the middle-stage. As shown in Table 4, the distribution pattern of C^{14} among the fermentation products gave evidence that lactic acid fermentation from pentose by lactic organisms did not involve carbonate fixation reaction, but carbonate was apparently fixed into other ether-soluble non-volatile acid which was not yet identified. From these findings, we may conclude that carbonate has no effect on lactic acid production from pentoses by lactic organisms except that it merely acts as a neutralizer.

Table 4. Incorporation of Carbon Atom from Carbonate into the Fermentation Products.

Strain	*S. faecalis*		*L. thermophilus* T_1	
Substrate	L-Arabinose		D-Xylose	
$NaHC^{14}O_3$		613,440 (c.p.m.)		8,020,000 (c.p.m.)
Initial sugar	1.27 (g)		1.90 (g)	
Consumed sugar	0.75		1.78	
Lactic acid*	0.56	1,400	1.34	145,600
Zn-lactate**		0		0
Formic acid	0.04	0	0.12	0
CO_2***		512,310	0.34	5,400,000
Residue		29,600		(not measured)
Recovered C^{14}		541,910		

* Ether-soluble non-volatile acid was calculated as lactic acid.
** Zn-lactate was prepared in a crystalline form.
*** CO_2 was liberated from carbonate by acid-formation.

3) *Lactic acid fermentation by the intact cells grown in media containing pentoses.*

As was shown in Table 1, lactic acid was produced in a yield of about 87% by weight of pentose utilized, a small amount of formate being formed at the same time as a by-product by *S. faecalis*. The proportions of formate to lactate were, however, varied by the conditions of fermentation. As the systems employed in the above culture experiments seem to be unappropriate to discuss the real fate of pentose metabolism, we must employ a more simplified experimental technique to meet this purpose. Thus, we carried out lactic acid fermentation anaerobically using the intact cells which were grown in the media containing pentoses.

As shown in Tables 5 and 6, the cells of *S. faecalis* grown in L-arabinose medium produced lactic acid in a yield of 95% by weight of the consumed L-arabinose under anaerobic condition and the cells of *L. thermophilus* T_1 grown in D-xylose medium converted 93% of consumed D-xylose to lactic acid under the same condition, but no detectable formation of formate was

observed in each case. CO_2 evolution and glycerol formation were both undetectable, however, small amounts of L-xylulose, L-ribulose and D-xylose were formed by *S. faecalis*, and D-xylulose by *L. thermophilus* T_1. O_2 consumption and CO_2 evolution from L-arabinose were observed when intact cells of *S. faecalis* were used aerobically. Studies on ketose formation and pentose metabolizing system under aerobic condition will be published in the next paper.

Table 5. Fermentation of Pentoses by the Intact Cells of *S. faecalis* and *L. thermophilus* T_1.

Strain	*S. faecalis*		*L. thermophilus* T_1			
Substrate	L-Arabinose		D-Xylose		L-Arabinose	
	50.0 mg	333.3 μM	50.0 mg	333.3 μM	50.0 mg	333.3 μM
Consumed sugar	50.0	333.3	42.0	280.0	36.0	240.0
Lactic acid	47.5	530.0	39.1	434.4	32.0	355.6
Formic acid		<20		<20		——
Yield of lactic acid mol./mol.	1.59		1.55		1.48	

mol./mol.: formed lactate μM per μM of consumed substrate.
For example, 1.667 is theoretical yield by formula (II).
Reaction mixture: cell suspension (ca. 60 mg dried cells) 5 ml;
buffer solution (M/10 phosphate, pH 7.0) 5 ml;
substrate (crystal) 50 mg;
total volume 10 ml
Reaction period: 18 hours

Table 6. Gas Balance in the Fermentation of L-Arabinose by the Intact Cells of *S. faecalis*.

Gas phase	N_2	Air
CO_2 evolution μM	0	5.58
O_2 consumption μM	0	5.02
Formed lactate μM	6.66	2.88

Reaction mixture: cell suspension (ca. 15 mg dried cells) 0.5 ml
buffer solution (M/15 phosphate, pH 7.0) 0.5 ml
L-arabinose 5 μM 0.2 ml
dist. water 0.8 ml
total volume 2.0 ml
Reaction period: 1 hour
Reaction temperature: 35°C
Gas balance were measured manometrically.

From these findings, we wish to propose the following fermentation formula (II) for the anaerobic pentose metabolism by *S. faecalis* and *L. thermophilus* T_1.

$$6\ C_5H_{10}O_5 \longrightarrow 10\ CH_3CHOHCOOH \qquad (II)^*$$

The above formula could be accounted for only by a recycling of carbon, possibly through pentose phosphate pathway, for regeneration of C_3-compound as a lactate precursor.

4) *Comparative studies on fermentation of glucose, pentoses and sedoheptulose by S. faecalis and L. thermophilus* T_1

As *S. faecalis* and *L. thermophilus* T_1 are so called homo lactic acid fermenters, so the theoretical yields of lactic acid from glucose by these organisms must be amounted to 100% of consumed sugar. Actually, however, lactate is not produced theoretically because lactate formation is accompanied with cell-growth and other by-paths in fermentation. For example, as shown in Table 7, the yield of lactic acid from glucose by *S. faecalis* and *L. thermophilus* T_1 was 87.5% and 90.0% respectively, and in both cases formate formation was observed but not acetate production. Comparison of the above data with the results obtained from pentose fermentation may suggest that there is no noticeable difference between the fermentations of pentoses and those of glucose in *S. faecalis* and *L. thermophilus* T_1.

Table 7. Comparison of Fermentation upon Glucose, Pentoses and Sedoheotulose by *S. faecalis* and *L. thermophilus* T_1

Strain	*S. faecalis*			*L. thermophilus* T_1		
Substrate	Glucose	L-Arabinose	Heptulose	Glucose	D-Xylose	Heptulose
Initial sugar (g)	3.45	2.32	0.22	2.80	2.80	0.22
Consumed sugar (g)	3.45	2.32	0.22	2.80	2.80	trace
Lactic acid (g)	3.02	1.91	0.20	2.52	2.18	
Formic acid (g)	0.13	0.10	0.04	0.13	0.21	
Acetic acid (g)	0	0	0	0	0	
Yield of lactic acid (%)	87.5	82.3	90.9	90.0	77.9	
Recovery of carbon (%)	92.1	87.9	109	96.0	87.1	

Sedoheptulose medium was 25 ml.

Supposing the new pentose metabolizing system of lactic organisms involves the so-called pentose phosphate pathway[13], sedoheptulose-7-phosphate and hexose-6-phosphate should be formed as intermadiates. These compounds were not detected by paperchromatography throughout our fermentation processes. It may be reasonably assumed that these compounds are so reactable that they are unable to accumulate in detectable amounts.

* The pentose metabolism by formula (II) may be designated homo lactic acid fermentation upon pentose just like that upon hexose.

The abilities of fermenting sedoheptulose by various strains were also examined. As the results shown in Tables 7 and 8, *S. faecalis* and *S. glycerinaceus* readily fermented sedoheptulose just as glucose, giving 90% yield of lactic acid and small amount of formate from consumed sugar. On the other hand, *L. thermophilus* T_1 could not produce any significant amount of acid in spite of its utilizing ability upon sedoheptulose (see Table 8).

Table 8. Utilization of Sedoheptulose by Lactic Acid Bacteria.

Strain	Acidity	Cell-growth
S. faecalis	1.00	##
S. glycerinaceus	0.10	+
S. liquefaciens	0.95	##
S. faecalis B06	0.35	+
L. arabinosus	0	(+)
L. thermophilus T_1	0.10	+
L. brevis	0	(+)
Leuc. mesenteroides B07	0	(+)

Acidity: number of N/10 NaOH ml per 2 ml of broth containing 1% sedoheptulose after 4 days' culture.
Cell-growth: ## good growth (normal growth).
+ moderate growth.
(+) slight growth.

These results may suggest that *S. faecalis* can ferment pentoses and sedoheptulose through the pentose phosphate cycle while *L. thermophilus* T_1 metabolizes pentose according to another pathway.

DISCUSSION

Clear evidence of the presence of a new pathway differing from the Fred-Peterson-Anderson scheme in the fermentation of pentoses by adapted cells* of *S. faecalis* and *L. thermophilus* T_1 was given from the studies of substantial balance in fermentation and tracer experiment. *S. faecalis* was also capable of fermenting sedoheptulose with the production of lactic acid in a yield of 90% by weight of consumed substrate. Therefore, the pentose metabolizing system of *S. faecalis* may be presented as follows:

6 Pentose ⟶ (Pentose-5-P ⟶ Heptulose-7-P ⟶ Hexose-6-P)
⟶ 10 Lactate

or,

* Recently, we have concluded that the formation of new pentose metabolizing systems in lactic acid bacteria are adaptively induced.

$$6\ C_5H_{10}O_5 \longrightarrow 10\ CH_3CHOHCOOH \qquad (II)$$

In this scheme, a carbon recycling system, possibly the pentose phosphate pathway, and Emoden-Meyerhof scheme may be involved. Consequently, pentoses may probably be metabolized via hexose-6-P. In the case of *L. thermophilus* T_1, although sedoheptulose was assimilated it was not fermented. So it is assumed that lactate might be produced via hexose-6-P through the other cyclic pathway of pentose, but not via sedoheptulose-7-P. Recently, E. Racker and E. Schroeder[14] reported that ribose-5-P was converted to fructose-6-P through octulose-8-P by transaldolase. There are some possibilities for the existence of a new cyclic pathway of pentose in *L. thermophilus* T_1. But in order to establish such a pathway, further investigation should be carried out.

SUMMARY

1) Pentose fermentation by various lactic acid bacteria can be classified into the following two types: a) Formula (I) which proceeds according to the Fred-Peterson-Anderson system, and b) formula (III) which supposedly involves a new pathway of pentose metabolism. Almost all of the examined organisms metabolized various pentoses according to either formula (I) or (III) exclusively. But, some exceptional strains fermented different pentoses by different schemes. For example, *Leuconostoc mesenteroides* B07 fermented various pentoses only according to formula (I) and *L. thermophilus* T_1 according to formula (III) exclusively. Whereas, *L. thermophilus* II was able to ferment L-arabinose by formula (I) and D-xylose by formula (III).

2) From comparative study of pentose fermentation in the presence and absence of carbonate and an isotopic experiment for incorporation of carbonate into lactate, it was concluded that carbonate fixation reaction is not involved in lactate formation from pentoses.

3) Anaerobically, the cells of *S. faecalis* grown in a L-arabinose medium produced lactic acid from L-arabinose in a yield of 95% by weight of consumed sugar, and likewise those of *L. thermophilus* T_1 grown in D-xylose medium converted 93% of consumed D-xylose to lactic acid. In both cases no formate and CO_2 were formed. As a consequence of these experiments, we proposed a new formula (II) as an alternative scheme of (III).

4) From the analysis of yields of lactate from glucose, pentose and sedoheptulose, no noticeable difference in yield among these fermentations was observed. Consequently, we have concluded that so-called pentose phosphate pathway may have no inconsistency for accounting for the pentose metabolizing system of *S. faecalis*. This hypothesis, however, can not be applied for such a strain like *L. thermophilus* T_1 which unable to ferment sedoheptulose.

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STUDIES ON THE CONIDIAL COLOR CHANGE IN ASPERGILLUS FUNGI

PART II. THE ROLE OF COPPER AND HALOGEN IONS IN THE FORMATION OF GREEN CONIDIA

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In spite of the well known fact that many species of *Aspergillus* fungi[1~4] require copper ion for the development of either green or black colors of conidia, little is known of the mechanism through which copper ion induces the color development. In a previous paper[5], the present author has already pointed out that the yellow mutants derived from *Aspergillus sojae* 260 and *Aspergillus oryzae* K. B. could produce green conidia only when these strains were cultivated in a medium containing halogen ions in addition to copper ions.

In connection with this, the role of copper and halogen ions in the development of green color of conidia was studied more extensively, the results appearing below.

MATERIALS AND METHODS

1. *Strains*

Aspergillus sojae 260[6] and its yellow mutant (Y_1)[7] were mainly used throughout this experiment. The former is a wild strain with Javel-green conidia, while the latter is a color mutant induced from the former by ultraviolet light irradiation. The color of conidial head is Yellow-ocher.

Besides these, two albino mutants (Al_1 and Al_4)[7] and one brown mutant (B_1)[7] of *Asp. sojae* 260 were also used in some experiments. The conidial color of these strains was quite stable even in sequential transfers on Koji-extract, malt-extract and Czapek-dox media.

2. *Preparation of conidial ash and mycelial mat ash*

The basal composition of the medium employed for the production of conidia and mycelial mats was: glucose, 3%; $(NH_4)_2HPO_4$, 0.1%; KH_2PO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, each 10^{-5} M. The initial pH was 5.4.

To meet the requirement of each experiment, the medium was modified a little by the addition of halogen ion as seen in Tables appearing later.

a) *Conidial ash and its extract.*

A mass production of conidia was carried out by the following method.

After piling three sheets of filter paper (Tōyōroshi No. 27) (from the bottom to the top) and adding a medium to an enamelled vessel ($24 \times 20 \times 4\,cm^3$ in volume) covering it with Japanese paper on the vessel, 5 ml. of conidial suspension (about 10^7 conidia per ml.) were sprayed on the surface. The vessel was placed in a moistry chamber for 5 days at 30°. When the surface was perfectly covered with matured conidia, the papers were then separated from the medium, and dried in a chamber with $CaCl_2$ as the desiccating agent for 3 days. The conidia on the paper were carefully collected in a manner so as to free them from mycelia and stored in a freezer at −20°. The yield of conidia was about 200 mg. per each vessel.

Taking 0.2 to 0.5 g. conidia into a dish, they were thoroughly burnt in a muffle at 450°–550°, for about 10 hours. During the course of heating, 0.5 ml. of conc. HNO_3 solution were dropped into a dish to promote ash-making. The ash thus prepared, was first treated with 1 ml. of HCl solution (1:1), and then after the addition of 5 ml. redistilled water, heated gently, so that the copper compound in the ash might be dissolved in the acidic water.

b) *Mycelial mat ash and its extract.*

Each strain was inoculated into 120 ml. of various media (see each Table) in 200 ml. Erlenmyer flasks, and incubated for 5 days at 30°. When mycelial mats were formed, they were washed three times with redistilled water and then dried. The preparations of ash and its extract were conducted in the same way as described above.

3. *Determination of copper contents*

Throughout this experiment, glasswares rinsed with chromic acid-H_2SO_4 solution and sequentially washed several times with redistilled water were employed. Copper contents in the conidial ash and mycelial mat ash extracts were measured by the modified Kimura et al. method[8] as follows:

After the addition of 5 ml. of 10% sodium citrate solution to the ash extract, it was adjusted to pH 9.0 with conc. ammonia solution. Again, adding 5 ml. of carbon tetrachloride containing dithizone in a concentration of 0.02%, the resulting mixture in a separatory funnel was shaken vigorously for 2 minutes. The water-insoluble carbon tetrachloride was transfered to an another separatory funnel, and in order to separate copper from other metals, the funnel upon the addition of 10 ml. of 0.01 N HCl solution, was shaken again for 2 minutes. This procedure was repeated for three times. For reason that copper salts in the ash extract should be contained in the carbon tetrachloride fraction, the fraction was evaporated in a silica dish on a water bath. The residue was first moistened with 0.5 ml. of NHO_3 solution (1:1) and then heated to destroy the organic matters. After it became cool, 0.5 ml. of HCl solution (1:1) were added to it, so that cupric oxide in the dry matter might be extracted by the acid. The extract upon the addition of 5 ml. redistilled water, was transfered to a separatory funnel and adjusted to pH 10.0 with conc. ammonia solution. Upon it, 2 ml. of 4% hydroxylamine solution and 10 ml. of 0.1% sodium diethyldithiocarbamate solution were added

in sequence in order to develop a golden-yellow color.

The colored substances were extracted with 5 ml. carbon tetrachloride and after filling up to 10 ml. with the same solvent, the extinction coefficient was measured by a Coleman-type photometer using a 430 mμ filter. Values obtained by the repetition of the above mentioned processes deviated in a range less than 5%.

RESULTS

1. *Copper contents of mycelial mats cultivated in the presence or absence of copper and chlorine ions*

At the time of this study, it had already been known that the wild type strain (*Asp. sojae* 260) requires copper ion alone and the yellow mutant of the same fungus requires both copper and chlorine ions for the formation of green conidia, and, in addition, that green or black strains of *Aspergillus* fungi contain, in general, a larger amount of copper in their mycelia than white or yellow strains. For this reason, the effect of chlorine ion upon the uptake of copper from the culture medium to the mycelial mat was studied, i.e., the wild strain and the mutant (Y_1) were cultivated on four kinds of media with or without supplemented copper and/or chlorine compounds.

Table 1. Copper Contents of the Mycelial Mats of Parent Strain and Y_1 Strain Harvested from Various Media.

Strain	Medium		Color of conidia	Cu contents of whole mycelial mat (γ)	Cu contents of whole culture filtrate (γ)	Cu uptake %*	Cu contents per g. dried mycelial mat (γ)
	Cu (10^{-5}M)	KCl (10^{-1}M)					
Asp. sojae 260 (parent strain)	−	−	Yellow	Trace	—	—	—
	+	−	Green	46.3	28.6	62.5	92.3
	+	+	Green	56.4	21.0	76.2	99.2
	−	+	Yellow	Trace	—	—	—
Asp. sojae 260 yellow mutant Y_1	−	−	Yellow	Trace	—	—	—
	+	−	Yellow	22.5	50.6	30.4	46.8
	+	+	Green	49.5	24.2	66.9	100.9
	−	+	Yellow	Trace	—	—	—

* Cu uptake $\% = \frac{\text{Cu contents } (\gamma) \text{ in mat}}{\text{Cu contents } (\gamma) \text{ in medium before incubation}}$.

As a result, it was demonstrated as seen Table 1 that the mycelial mats with green conidia contained a larger amount of copper than the mycelial mat with yellow conidia. Besides, it was also characteristic that the uptake of

copper by the mutant (Y_1) was greatly accelerated by the presence of chrorine ions in contrast with the wild strain which was not almost affected by chlorine ions. This specificity will be discussed later.

2. *The effect of different concentration of chlorine ion upon the copper uptake by the mutant Y_1*

The effect of chlorine ion upon the uptake of copper by the mutant (Y_1) was studied extensively.

Table 2. Copper Uptake in the Presence of Different Concentrations of Chlorine Ion (Y_1 strain).

No.	Medium		Greenization *	Cu contents of whole mycelial mat (γ)	Cu contents of whole culture filtrate (γ)	Cu uptake %	Cu contents per g. dried mycelial mat (γ)
**	Cu 10^{-5}M	KCl concentration M					
A	+	−	−	12.9	59.4	18.4	27.7
B	+	1/100	±	19.3	49.5	27.6	37.1
C	+	1/50	+	24.2	42.9	34.6	48.9
D	+	1/20	++	28.6	41.6	40.9	60.3
E	+	1/10	+++	38.5	29.0	55.1	82.3
F	+	1/5	+++	45.1	24.4	64.4	99.2

* Degree of greenization: see this Journal **3**, 125 (1957).
** These signs are the same as those shown in Plate 1.

As seen in Table 2, the conidial color changed into deeper green with the increase of chlorine ion concentration, and in the same way, the copper uptake ratio increased with the chlorine ion concentration, suggesting that there may be a close relationships among the degree of greenization, the concentration of chlorine ion, and the uptake of copper.

3. *Enhancement of copper uptake by other halogen ions*

In this experiment, chlorine ion was replaced either by bromine ion or iodine ion.

Table 3. Effect of Bromine and Iodine Ions upon the Copper Uptake by Y_1 Strain.

Kind of halogen salt*	Cu contents of whole mycelial mat (γ)	Cu contents of whole culture filtrate (γ)	Cu uptake %	Cu contents per g. dried mycelial mat (γ)
Control (no addition)	14.4	39.8	20.0	28.3
KCl	52.2	18.0	72.5	112.2
KBr	41.6	18.0	57.7	94.5
KI	49.6	20.0	68.9	148.0

* The final concentration of each halogen ion is 1/10 M.

As shown in Table 3, both ions promoted the copper uptake in the same order as chlorine ion, but no stimulative effect was observed, when they were replaced by anions such as sulphate, nitrate or phosphate etc.

4. *Copper uptake by other mutants*

In order to know whether or not other color mutants could take up copper in the absence of halogen ion, four or five strains shown in Table 4 and 5 were cultivated on a halogen ion-deficient medium. As shown in the tables, the contents of copper in both the mycelial mat and the conidia of these strains were considerably larger as compared with that in the yellow mutant (Y_1). The uptake of copper by these color mutants in the presence of halogen ions was not examined, but it must be noted that these albino or brown mutant do not turn into green even in the presence of halogen ions as reported previously.[5]

Table 4. Comparison of Copper Uptake in Various Color Mutants.

Strain	Copper uptake %	Copper contents per g. dried mycelial mat (γ)
Asp. sojae 260 parent strain	61.1	76.8
〃 〃 〃 yellow mutant Y_1	32.0	45.0
〃 〃 〃 brown mutant B_1	63.4	101.8
〃 〃 〃 albino mutant Al_1	38.5	51.0
〃 〃 〃 〃 〃 Al_4	47.7	75.9

Table 5. Copper Contents in Conidia.

Strain	Color of conidia	Copper (γ)	Mean of Cu contents per g. dried conidia
Asp. sojae 260 parent strain	Oil green	22～35	25.8
〃 〃 〃 yellow mutant Y_1	Apricot yellow	15～18	17.2
〃 〃 〃 brown mutant B_1	Sudan brown	19～24	22.4
〃 〃 〃 albino mutant Al_1	White	15～22	19.0

5. *Detection of copper and other metals by paper chromatography*

Finally, the metal components of the ash extracts prepared from the mycelial mats cultured on a halogen deficient medium were examined paper-chromatographically according to Kikkawa et al. method.[9]

As illustrated in Fig. 1, the presence of copper was demonstrated in all the strains. The copper contents of mats of brown and albino (Al_4) strains were significantly less than those of green strains, but it was much higher than that of yellow mutant (Y_1). As for reference, the spots corresponding to Fe and Ni are also illustrated.

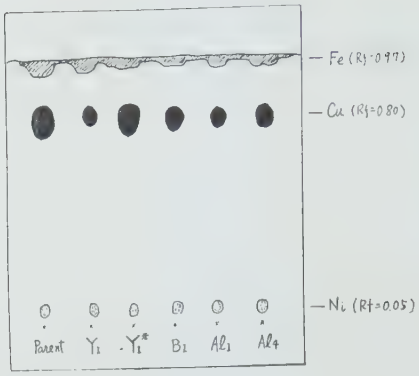


Fig. 1. Paper Chromatogram of Ash Extract (mycelial mat).
Solvent: a mixture of *n*-butanol, acetone and HCl (4:10:2).
Reagent: 0.2% rubeanic acid-alcohol solution.
Color: Cu—dark green, Fe—brown and Ni—blue.
* This sample is mycelial mat of Y_1 strain with modified green conidia.

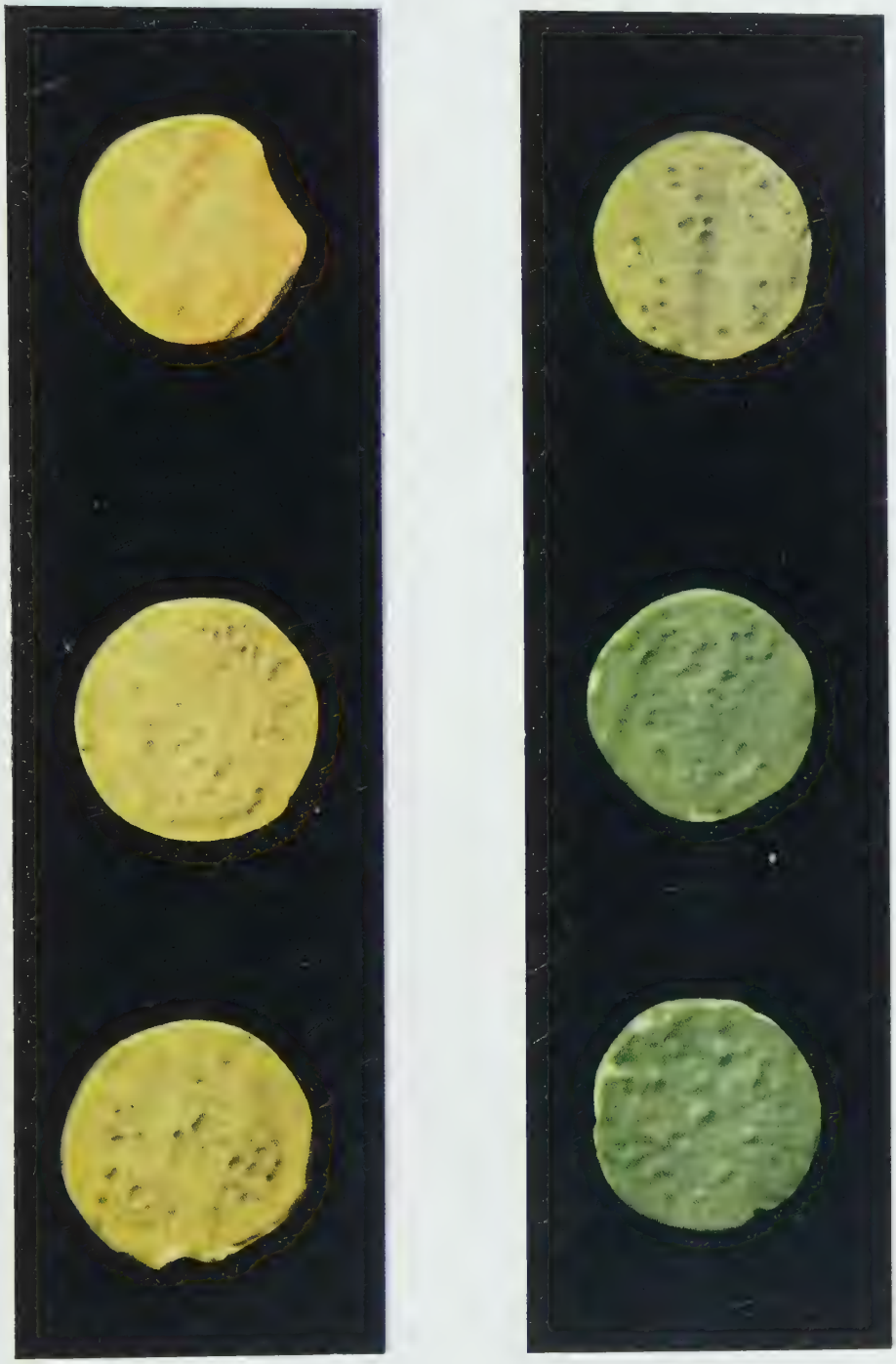
DISCUSSION AND CONCLUSIONS

As described in a previous paper[5], the yellow strain (Y_1) seemed to be a mutant in which an intermediate step between yellow and green conidia formation was genetically blocked.

In this paper, it has been discovered that the strain can take up copper in an amount sufficient to produce green conidia only when halogen ion is supplemented together with copper ion (Table 1). In addition, it was also found that green conidia contain a larger amount of copper than others (Table 4), and that the strains with brown or albino conidia take up a larger amount of copper than the yellow mutant (Y_1) when they are cultivated in a medium lacking halogen ion.

Grounding on these facts, it may be concluded that copper ion plays an important role in the development of green color, and that the wild strain is healthy with regard to its copper uptake system, as it produces green conidia in the presence of copper ions. While the system is not perfect in the yellow mutant on account of genetic event, the strain can not take up enough amount of copper even in the presence of copper ions. Then, in consideration of the role of halogen ion in green conidia formation, it was found that the ion stimulates the copper uptake of yellow mutant. It is, therefore, evident that the ion does act in such a manner as to restore the genetic disturbance taking place in the copper uptake system, but the real mechanism of the action remains still unknown. It may, of course, also be possible to assume that the halogen ion is essential for the copper uptake in yellow mutant, and the disturbance which takes place in the halogen relating system might be considered as the real course in the yellow color development.

PLATE 1. Conidial Color Change of Yellow Mutant (Y_1) with Gradated Concentrations of Chloride.



A (Upper left):—On medium lacking halogen ion.

B (Center left):—On medium containing 1/100 M. KCl

C (Lower left):—On medium containing 1/50 M. KCl

D (Upper right):—On medium containing 1/20 M. KCl

E (Center right):—On medium containing 1/10 M. KCl

F (Lower right):—On medium containing 1/5 M. KCl

All media contain 10^{-5} M. $CuSO_4$.

To define the role of copper in green color development, the author is now investigating whether or not copper is the component of the green pigment.

SUMMARY

By cultivating a green strain and a yellow mutant in media lacking copper and/or halogen ions, the responsibility of these ions upon the development of green color of conidia was studied.

1. The green strain took up a larger amount of copper into its mycelia when it was cultivated in a medium containing copper ion.

2. The yellow mutant could take up a sufficient amount of copper to produce green conidia only when halogen ion was supplemented together with copper ion.

3. By a comparison test with other color mutants, the yellow mutant was proved to be extremely poor in the ability of copper uptake.

On the ground of these bases, it was concluded that the development of green color of conidia in these strains is closely related with the ability of copper uptake and that halogen ion can restore the genetic defect in the copper uptake system.

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THE FERMENTATIVE PRODUCTION OF L-ORNITHINE (Preliminary report)

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In the previous communications[1),2)], the authors have reported on the accumulation of a large amount of L-glutamic acid from carbohydrate and ammonia source by *Micrococcus glutamicus*. During the course of mutation studies of a strain of this organism, an amino acid other than glutamic acid was found to be accumulated by an arginine or citrulline requiring-mutant strain. The amino acid accumulated was proved to be L-ornithine by isolation and purification of the fermentation product. L-ornithine, which is known as one of the important intermediates of so-called "ornithine cycle" and arginine biosynthesis, has heretofore never been produced in such a large amount by microorganisms.

The fermented broth, which was examined by paper chromatography and confirmed to contain only one amino acid exclusively, was treated with a strong acidic cation exchange resin, Amberite IR-120, and the amino acid absorbed was eluted with 2N HCl. The eluate was concentrated and adjusted to approximately pH 6. After the addition of absolute ethanol in the cold, a fine crystalline precipitate was obtained. This crystal was purified by recrystallization and active carbon treatment. The amino acid thus obtained gave the same Rf value of an authentic ornithine on a paper chromatogram, and melted at 235–6°C. *Anal.* Calculated for $C_5H_{13}O_2N_2Cl$: C, 35.56; H, 7.77; N, 16.60. Found: C, 35.49; H, 7.40; N, 16.67. $[\alpha]_D^{20}$; +10.9° (C=5.5, H_2O). These analytical data are in accordance with the literature values of L-ornithine. The expected amount of CO_2 was also evolved from a sample solution by an L-ornithine decarboxylase preparation of *Clostridium septicum* P. III (NCTC No. 547)[3)].

The fermentative production of L-ornithine was successfully conducted as follows: the medium contained 10% glucose, 0.1% K_2HPO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 1.0% NH_4Cl, 0.3% urea, 0.5% corn steep liquor, 1.0% NZ-amine, 0.0015% phenol red. The pH was adjusted to 7. Twenty ml of this medium was dispensed in a 250-ml Erlenmeyer flask and inoculated with 5% of inoculum broth. The organism was cultured at 28°C on a rotary shaker. The pH of the culture medium was maintained at a state of neutrality with the addition of a small amount of 20% urea solution. The course of this fermentation is presented in Table 1. The quantitative determination of ornithine was carried out by the method of Chinard[4)].

As shown above, about 0.36 mole of L-ornithine was produced from 1

Table 1. The course of L-ornithine fermentation.

Incubation time	Glucose	L-Ornithine	L-Glutamic acid	α-Ketoglutaric acid	Lactic acid
hours	%	mg/ml	mg/ml	mg/ml	mg/ml
0	10.0	—	—	—	—
24	5.48	10.0	0	1.2	5.0
48	1.77	24.0	0.23	1.5	1.8
72	0.54	26.2	0.1	2.5	1.1

mole of glucose. Because of this extremely high yield of ornithine production, the investigation of the mechanism of this fermentation is now in progress.

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